THE IMPACT OF PERIODONTAL INFECTION AND ITS TREATMENT ON SYSTEMIC INFLAMMATION, OXIDATIVE STRESS AND ENDOTHELIUM INTEGRITY

Thesis presented for the degree of Doctor of Philosophy

in the Faculty of Medicine, University of London

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« "O frati," dissi, "che per cento milia
perigli siete giunti a l'occidente,
a questa tanto picciola vigilia

d'i nostri sensi ch'è del rimanente
non vogliate negar l'esperïenza,
di retro al sol, del mondo sanza gente.

Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza". »

Canto XXVI, Inferno (vv. 112-120)
Divina Commedia

Dante Alighieri (1265-1321)

DECLARATION

I, Marco Orlandi, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:  Date: 12.09.2016

ABSTRACT

Background: Periodontitis (PD) is a chronic inflammatory disease. The low-grade systemic inflammatory and oxidative status seems to be a plausible mechanism to explain the relationship between cardiovascular disease (CVD) and PD. The aim of this PhD was to investigate the association between PD and its treatment and the human vascular homeostasis.

Methods: 4 studies were conducted: 1) a systematic review and meta-analysis of cases with PD and controls to assess the differences in intima-media thickness and endothelial function between cases and controls, 2) a Randomized controlled trial investigating the effect of periodontal therapy on mitochondrial oxidative stress, systemic inflammation, endothelial function and metabolic control in patients with type II diabetes mellitus and periodontitis 3) a randomized controlled trial investigating the effect of periodontal treatment on the carotid intima media thickness 4) a RCT controlled trial investigating the effect of remote ischemic preconditioning on the endothelial dysfunction following periodontal treatment.

Results: Study I demonstrated that there is an association between PD, flow mediated dilation and carotid intima media thickness, measures of cardiovascular risk. Study II reported the benefit of periodontal therapy on the endothelial function, mitochondrial oxidative stress and glycemic control in patients with Type 2 diabetes and PD. In Study III we reported the beneficial effect of periodontal treatment on the carotid intima-media thickness and lastly in Study IV demonstrated the protective effect of remote ischemic preconditioning toward the acute endothelial dysfunction following periodontal treatment.

Conclusions: This PhD project provides evidence in support of the association between periodontitis and cardiovascular disease.

STATEMENTS OF CONTRIBUTIONS

The experimental and vascular data described in this thesis are the result of 3 years of collaborative work between the Periodontology Unit of the UCL Eastman Dental Institute, the Vascular Physiology Unit at the Institute of Cardiovascular Sciences as well as the Department of Biomaterials and Tissue engineering. All studies included in this thesis are original in concept and design. I performed most of the work outlined in this thesis but I would like to acknowledge the specific contribution of all staff and collaborators.

My contributions to the studies presented in this thesis were as follows:

Chapter 3.

I contributed to all steps from the development of the protocol to writing of the article; protocol writing, design and conduct of searches, retrieving and screening articles, data extraction, risk of bias assessment, evidence tables, meta-analysis, and manuscript preparation.

Contributors

Dr F. D'Aiuto: abstract screening, interpretation of results and manuscript preparation

Dr A. Petrie: statistical analysis and manuscript preparation

Dr J. Suvan: data extraction, bias assessment and manuscript preparation

Dr S. Masi: manuscript preparation

Prof A. Hingorani: manuscript preparation

Prof J. Deanfield: manuscript preparation

Chapter 4.

I contributed to the development of the protocol, protocol writing, delivering periodontal treatment in the study, blood sampling collection, laboratory analyses including LPS assays, PBMC isolation and staining, FACS acquisition of data, Flow-jo off line analysis, statistical analyses and data interpretation.

Contributors:

Mrs D. Bhowruth: acquisition and analysis of all the vascular data

Dr I. Kingston: Multiplex cytokines/biomarkers analyses

Mr M. Parkar and Dr s. Masi: development of the PBMC isolation and FACS analysis protocol

Dr J. Suvan: trial coordination and management

Dr F. D'Aiuto: protocol development and writing, statistical analyses and data interpretation

Eastman Investigation Centre (ECIC) staff: support for the conduct of the trial

Chapter 5

I contributed to the delivering periodontal treatment in the study, blood sampling collection and Multiplex assays, statistical analyses and data interpretation.

Contributors:

Mrs D. Bhowruth: acquisition and analysis of all vascular data

Dr I. Kingston: Multiplex analysis

Dr J. Suvan: Ethics application, trial coordination and management

Dr F. D'Aiuto: statistical analyses and data interpretation.

Eastman Investigation Centre (ECIC) staff: support for the conduct of the trial

Chapter 6.

I contributed to the development of the protocol, protocol writing, ethics application, delivery of periodontal treatment, blood sampling, laboratory analyses including d-ROM test, HEME0-1 ELISA and LPS assay, PBMC isolation and staining, FACS acquisition of data, Flowjo off line analysis, statistical analyses and data interpretation.

Contributors:

Mrs D. Bhowruth: acquisition and analysis of all the vascular data

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Dr J. Suvan: trial coordination and management

Dr F. D'Aiuto: statistical analyses and data interpretation

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PUBLICATIONS RELATED TO THIS WORK

1. D'Aiuto, F., **Orlandi, M.**, & Gunsolley, J. C. (2013). *"Evidence that periodontal treatment improves biomarkers and CVD outcomes*". *Journal of Clinical Periodontology, 40*, S85-S105.

2. **Orlandi, M.**, Suvan, J., Petrie, A., Donos, N., Masi, S., Hingorani, A., . . . D'Aiuto, F. (2014). *"Association between periodontal disease and its treatment, flow-mediated dilatation and carotid intima-media thickness: A systematic review and meta-analysis"*. *Atherosclerosis, 236 (1)*.

3. Masi, S.*, **Orlandi, M***., Parkar, M., Bhowruth, D., Donos, N., Kingston, I., O'Rourke, C., Deanfield, J., D'Aiuto, F. (2016) *"Mitochondrial oxidative stress production is modulated by systemic inflammation and tracks changes of endothelial function in patients with type II diabetes mellitus".* **Under authors revision. Manuscript in preparation**

*These authors contributed equally to this work

4. **Orlandi, M.,** Bhowruth, D., Masi, S,. Housenloy, D., Deanfield, J., D'Aiuto, F. (2016) *"Remote ischemic preconditioning (RIPC) modulates endothelial dysfunction, inflammatory and oxidative response in a human model of systemic inflammation".* **Under authors revision. Manuscript in preparation**

LIST OF ABBREVIATIONS

Abs	Antibodies
ACC	American College of Cardiology
AGEs	Advanced glycation end products
ACC	American College of Cardiology
AHA	American Heart Association
AMI	Acute myocardial infarction
apoA	Apolipoprotein A
apoB	Apolipoprotein B
BOP	Bleeding on probing
CCA	Carotid artery
CEJ	Cemento-enamel junction
CHD	Coronary heart disease
COX-2	Cyclo-oxygenase-2
CPT	Community periodontal treatment
CRP	C-reactive protein
CSF	Colony stimulating factors
CV	Cardiovascular
CVD	Cardiovascular disease
ELAM	Endothelial leukocyte adhesion molecule
EPC	Endothelial progenitor cells
ESR	Electron spin resonance
FGM	Free gingival margin
FRS	Framingham risk scores
GCF	Gingival crevicular fluid

H_2O_2	Hydrogen peroxide
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein cholesterol
HO1	Heme oxygenase-1
Hs-CRP	High sensitivity C-reactive protein
HSPs	Heat-shock proteins
ICA	Internal carotid artery
ICAM	Intercellular adhesion molecule
ICAM-1	Intercellular adhesion molecule 1
IEL	Internal elastic lamina
IFN	Interferons
IFN-γ	Interferons-γ
IL-18	Interleukin-18
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
iNOS	Inducible nitric oxide synthase
IPT	Intensive periodontal treatment
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LES	Systemic lupus erythematosus
LOX	Lipoxygenase
LP(a)	Lipoprotein(a)

LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein
MI	Myocardial infarction
MMPs	Matrix metalloproteinases
mtRS	Mitochondrial reactive species
NAP-1	Neutrophil attractant protein
NF-κB	Nuclear factor-κB
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
oxLDL	Oxidized LDL
PAD	Peripheral arterial disease
PAI-1	Plasminogen activator inhibitor-1
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PD	Periodontitis
PDL	Periodontal ligament
PV	Pulse velocity
REC	Recession
RNO	Reactive nitrogen species
ROM	Reactive oxigen metabolite
ROS	Reactive oxygen species
RR	relative risk
RS	Reactive species
SAA	Serum amyloid A
sCD40L	Soluble CD40 ligand

SRP	scaling and root planing
SPT	Supportive periodontal therapy
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TF	Tissue factor
TG	Triglycerides
TGF	Transforming growth factors
TGs	Triglycerides
Th1	T-Helper 1 cells
Th2	T-helper2 cells
TIMPs	Tissue inhibitors of metalloproteinases
TLRs	Toll-like receptors
TNF-α	Tumour Necrosis Factor-α
US	United States
VCAM-1	Vascular cell adhesion molecule 1
vLDLs	Very low-density lipoproteins
VSMCs	Vascular smooth muscle cells
vWF	Von Willebrand factor
WBC	White blood cell
WHO	World Health Organization

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1. Introduction

Periodontitis (PD) is a chronic inflammatory disease triggered by a dysbiosis of the subgingival microbiome and affecting the tissues supporting the dentition called periodontium[1]. This includes gingivae, periodontal ligament, root cementum and alveolar bone and they are all affected by the course of PD[2]. Its progression, depending on the level of severity and extent, may affect the stability, and the function of the involved dentition having an impact on the patient's quality of life and ultimately leading to tooth loss in its advanced forms. PD treatment relies mainly on the mechanical removal of the dental biofilm (pathogenic bacteria) from the root surfaces by means of hand and ultrasonic instruments performed on a regular basis[3]. Over the last 20 years, accumulating evidence supported an association of PD with multiple chronic diseases with an established etiologic inflammatory component including diabetes and cardiovascular disease suggesting the hypothesis of a potential contribution of PD in their onset and progression[4-6]. The systemic impact of the periodontal infection and its treatment has been investigated providing evidence on a number of putative mechanisms of association between PD and chronic diseases [7,8]. The immune response is known to be involved in both the defense and collateral damage of the periodontal tissues[9]. A characteristic systemic inflammatory profile associated with PD has been shown to be modulated by periodontal treatment (with acute inflammation increase in the short term and reduction of systemic inflammation over the long term)[10,11]. Periodontitis treatment, therefore represents not only a therapeutic procedure, including tissue damage and bacterial dissemination in the blood stream but an opportunity to study systemic inflammation in humans and its impact on various organs. It has been previously reported a two-fold impact of PD and its treatment on the vasculature homeostasis and a number of key processes

(inflammatory/oxidative) which are very relevant in the development and progression of vascular disease (atherosclerosis)[12].

The aim of this project was to investigate the magnitude of the association between PD and vascular integrity and explore the acute and chronic changes and pathways affected by periodontal treatment on systemic inflammation and vascular function.

1.1. Periodontitis

1.1.1. Periodontal tissues

The periodontium (peri = around, odontium = tooth) is formed by tissues "investing and supporting the teeth"[2]. Gingiva, periodontal ligament, root cementum and alveolar bone represent a functional unit (Figure 1). Its main purpose is to guarantee the attachment of the teeth to the jawbone preserving the masticatory function of the dentition. It is subject to changes due to the aging process and can be damaged by the inflammatory response triggered by periodontal bacteria during PD.

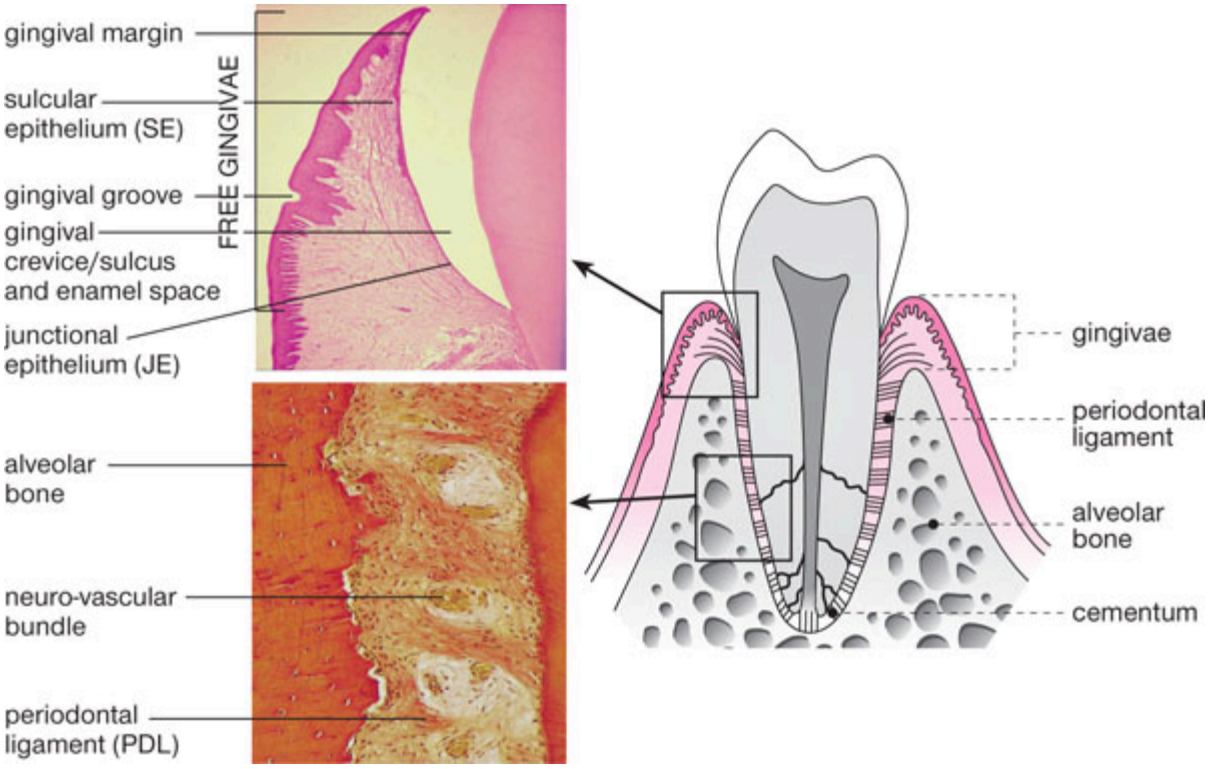


Figure 1 Periodontal tissues

1.1.1.1. Gingiva

Together with the surface of the hard palate it forms the region of the oral mucosa called masticatory mucosa covering part of the alveolar process and the cervical area of the teeth. Its width comprises the region from the gingival margin to the muco-gingival junction. The change in the tissue color from the coral pink of the gingiva to the dark red of the apical alveolar mucosa contribute to their distinction. Based on the location, it can be categorized in marginal, attached and interdental gingiva and histologically it consists of two different layers: epithelium and connective tissue.

1.1.1.1.1. Gingival epithelium

The gingival sulcus is an invagination between the marginal gingiva and the dentition and according to the relationship with teeth, three different epithelial layers can be identified:

- oral, (keratinized, stratified, squamous) facing the oral cavity.
- sulcular,(non keratinized, stratified, squamous) facing but not in contact with the dentition.
- junctional, (non keratinized, single layer) attached to the calcified dental tissues (enamel or cementum).

Different cell populations can be detected in the epithelial layer; keratinocytes, representing the majority, melanocytes, Langerhans cells, Merkel's cells and inflammatory cells. The gingival sulcus represents the starting point of the periodontal infection characterized by a microbial accumulation and related host immune response.

1.1.1.1.2. Gingival connective tissue

The connective tissue compartment accounts for the majority of the gingival volume and is represented by collagen organized in fiber bundles forming the gingival

ligament, micro-vasculature, glycoproteins and proteoglycans. Its cellular component mainly comprises fibroblasts and leukocytes.

1.1.1.2. Periodontal ligament (PDL)

It is a complex of collagen fibers located in the tight space between the dentition roots and the *lamina dura*, the alveolar bone interfacing the teeth. Its width ranges from 0.2 to 0.4 mm. Its main function is to absorb and distribute within the alveolar bone the forces applied to the teeth. The dentition mobility depends on the state of the PDL. Within its structure, it also comprises nerve fibers and different groups of cells: fibroblast, osteoblast, osteoclast, cementoblast, epithelial cells.

1.1.1.3. Root cementum

It is a mineralized tissue covering the teeth root surface and involved in its bond with the periodontal ligament. The root cementum is also involved in the root repair after damage. Its content comprises a high mineral component (65%) represented by hydroxyapatite and has a cellular component consisting of cementocytes. Cementoblasts of the PDL produce different cementum layers distinguished by their histological properties. The cementum undergoes new layer apposition within the ageing process becoming thicker throughout life. The fibers of the periodontal ligament are subject to a mineralization process becoming embedded in the cementum contributing to the attachment apparatus of the dentition to the bone.

1.1.1.4. Alveolar bone

It represents the osseous component of the dentition attachment apparatus. The alveolar bone is a district of the mandible and maxilla forming the teeth alveoli and its development depends on their eruption and persistence. The cortical bone represents the walls of the sockets surrounded by trabecular bone meanwhile the alveolar bone

proper interface the dentition. Its resorption due to inflammatory periodontal processes affects the dentition stability and position.

1.1.2. Classification

Since the 1920, different classifications of the diseases affecting the periodontium have been proposed. Their evolution was driven by the request for a higher precision in the definition of different clinical conditions. The classification proposed in 1999 by the American Academy of Periodontology, even if it states that "The present knowledge of the periodontal disease seems to be insufficient to classify them on the basis of host/infection paradigm", is the most widely accepted and used for most of research and academic purposes[13] (Table 1).

Table 1 Classification of periodontal diseases[14]

II. Chronic Periodontitis†	V. Necrotizing Periodontal Diseases
A. Localized	A. Necrotizing ulcerative gingivitis (NUG)
B. Generalized	B. Necrotizing ulcerative periodontitis (NUP)
III. Aggressive Periodontitis†	VI. Abscesses of the Periodontium
A. Localized	A. Gingival abscess
B. Generalized	B. Periodontal abscess
IV. Periodontitis as a Manifestation of Systemic Diseases	C. Pericoronal abscess
	VII. Periodontitis Associated With Endo lesions
A. Associated with hematological disorders	A. Combined periodontic-endodontic lesions
1. Acquired neutropenia	
2. Leukemias	
3. Other	
B. Associated with genetic disorders	
1. Familial and cyclic neutropenia	
2 Down syndrome	
3. Leukocyte adhesion deficiency syndromes	
4. Papillon-Lefèvre syndrome	
5. Chediak-Higashi syndrome	
6. Histiocytosis syndromes	
7. Glycogen storage disease	
8. Infantile genetic agranulocytosis	
9. Cohen syndrome	
10. Ehlers-Danlos syndrome (Types IV and VIII)	
11. Hypophosphatasia	
12. Other	
C. Not otherwise specified (NOS)	

1.1.2.1. Chronic periodontitis

Chronic periodontitis is the most common form of periodontal disease. It differs from gingivitis since it involves the irreversible disruption of the periodontal tissues. It is considered an evolution of a preexistent gingival inflammation. However, the lower incidence compared to gingivitis suggests a host predisposition to its onset.

Its clinical features are described in Table 2[13].

Table 2 Clinical features of chronic PD
Alteration in the color, texture and volume of the marginal gingiva
Increased gingival sulcus depth or presence of periodontal lesions
Bleeding on probing (BOP)
Loss of attachment level
Gingival margin recessions
Loss of alveolar bone
Root dentition exposure
Increased teeth mobility
Drifting or teeth loss

In addition, it has the following characteristics:

- Higher prevalence in adults
- Its extent and severity are related to predisposing risk factors such as oral hygiene, smoking, stress and systemic risk factors such as diabetes
- Presence of a subgingival biofilm hosting different bacterial species depending on subjects and sites
- Detection of subgingival calculus on the surface of the diseased dentition
- Is defined as localized or generalized based on a pragmatic threshold (30%) of the affected dentition

- The degree of probing attachment loss (PAL) identifies 3 category of severity
    - mild (PAL = 1–2 mm)
    - moderate (PAL = 3–4 mm)
    - severe (PAL ≥5 mm)

- The host factors are responsible for the onset and progression of the disease

- Slow to moderate progression rate in the majority of the cases.

1.1.2.2. Aggressive periodontitis

Aggressive periodontitis is characterized by an early age of clinical manifestation, rapid progression and a tendency for familiarity[13] (Table 3).

Table 3 Aggressive PD features
Rare occurrence
Non-contributory medical history
Rapid attachment loss and bone destruction
Familial aggregation of cases

In addition the following features have been described:

- Amounts of microbial deposits inconsistent with the severity of periodontal tissue destruction

- Elevated proportions of Aggregatibacter actinomycetemcomitans

- Phagocyte abnormalities

- Hyper-responsive macrophage phenotype

- Potential for a self-arresting progression

According to the dentition involvement, it can be distinguished in:

- Localized aggressive periodontitis

    - Puberal onset.
    - Involvement of first molar/incisor with interproximal attachment loss on at least two permanent teeth, one of which is a first molar, and involving no more than two teeth other than first molars and incisors.

- Robust serum antibody response to infecting agents.

- Generalized aggressive periodontitis

  - Usually affecting persons under 30 years of age.
  - Generalized interproximal attachment loss affecting at least three permanent teeth other than first molars and incisors.
  - Pronounced episodic nature of the destruction of attachment and alveolar bone.
  - Poor serum antibody response to infecting agents.

1.1.3. Epidemiology

Across continents there are large differences in the reported prevalence of periodontal diseases including periodontitis[15,16]. Chronic periodontitis is a multi-factorial disease that affects a significant proportion of the general population, with more than 50-60% of individuals presenting with some forms of periodontitis. Severe PD is however less common, affecting approximately 5-15% of the dentate adult population. Differences in the methodology adopted for the diagnosis of periodontal diseases have contributed to the difficulty in defining its global prevalence. The assessment of periodontal tissues inflammation, presence of periodontal lesions and the loss of the dentition support have been evaluated with a variety of clinical indices. The lack of uniform criteria in epidemiological research reduces the validity of comparisons between multiple observational studies. The majority of the epidemiological studies have been performed in the developed world and just few of them have used systems allowing the estimation of a general trend. Today there is no definitive data on the prevalence of periodontal diseases worldwide. The Adult Dental Health Survey 2009 reported that 45% of the population in England presented with at least 1 periodontal lesion (probing pocket depth greater than 4mm) and with 8% having at least one pocket over 6 mm[17](Figure 2).

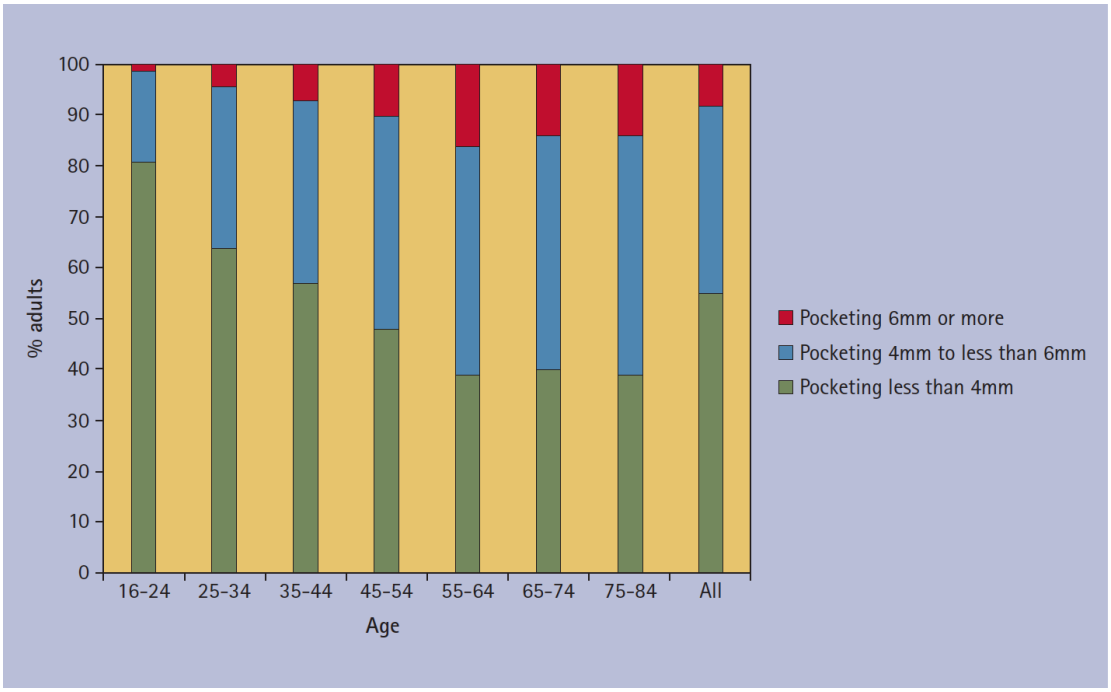


Figure 2 Adult Dental Health Survey data

Percentage of those with any periodontal pocketing at three levels of severity (y axis) in England, Wales and Northern Ireland by age group (x axis)

Data from the last update from the National Health and Nutrition Examination Survey (NHANES) indicated that 47% of US dentate adults aged 30 years and older had periodontitis, moderate and severe forms affected 38% of the adult population 30 years and older and 64% of adults 65 years and older[18].

1.1.4. Etiology

Dental plaque represents the main etiological factor responsible for the onset of periodontitis[19,20]. However the presence of a bacterial biofilm on the dentition triggers a reversible inflammatory process of the superficial periodontium, gingivitis, but is not necessarily related to the development of periodontal disease[21]. Therefore, there are several factors responsible for a different susceptibility observed in the general population; the amount and the composition of the dental plaque and the host response to its presence.

1.1.4.1. Microbiology of periodontal disease

Dental plaque consists of a biofilm containing more than 500 species of bacteria of which we have not cultured many species[22]. The presence of dental plaque is detectable after the dentition eruption. The initial colonization is lead by Streptococci and Actinomyces species. Within maturation of the biofilm there is a switch from mainly Gram positive aerobic bacteria to primarily Gram negative motile rods[23,24]. Extensive microbial research has aimed to identify a single or a group of pathogens directly responsible for the onset and progression of periodontitis. For the time being it is still not possible to draw conclusions on bacterial role in the etiology and pathogenesis of the disease. A classic experiment has reported the association of specific microbial species (Tannerella forsythia, Porphyromonas gingivalis, Treponema denticola), defined as the red complex, with the presence of disease[25]. Aggregatibacter actinomycetemcomitans has been also frequently detected specifically in aggressive forms of periodontitis[26]. The presence of the above species is reported in periodontal lesions and their elimination seems to be linked to a favorable prognosis after the treatment. In addition, their release of virulence factors give biological plausibility to a putative role in the development of the disease. Other species (Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros, Fusobacterium nuc. vincentii, Fusobacterium nuc. nucleatum, Fusobacterium nuc. polymorphum and Fusobacterium periodonticum. Eiknella nodatum, Campylobacter gracilis, Streptococcus constellatus and Capnocytophaga rectus), called the amber group, have been identified less frequently in sites of periodontal destruction[25]. The limitations to the microbial primary pathogenic role are that the species of the red and amber complex cannot be detected in all the periodontal lesions and they are also isolated from the healthy periodontium. Therefore it is still unclear how these bacteria interact with the host in

order to determine disease. In the past, observational studies reported the presence of periodontal lesions mainly in sites with presence of plaque and calculus[27]. Therefore the main etiologic hypothesis, defined the non-specific plaque hypothesis, was related to the volume of plaque and calculus accumulated on the diseased dentition. Experimental gingivitis models proved that plaque is the main factor responsible for gingivitis[28] and it was believed that periodontitis was the next step in the progression of the gingival inflammation. Subsequent observational studies on the natural progression of periodontal disease on laborers from tea estates in Sri Lanka demonstrated that in a population with poor oral hygiene and generalized plaque, calculus and gingival inflammation there were three groups responding differently to the same microbial trigger. The susceptibility to the disease, with no difference in the volume of plaque, was moderate in 81% of the population, severe in the 8%, meanwhile there was no development in 11% of the sample. As a consequence, the non-specific plaque hypothesis has been universally rejected introducing other factors such as the host inflammatory response to the dental biofilm[29]. Ongoing research is investigating the role of the immune response and the genetic susceptibility of some individuals to periodontitis.

1.1.4.1.1. Microbial dysbiosis

The Microbial shift hypothesis suggests a link between specific diseases and a shift in the composition of the local microbiota. Microbial shift or dysbiosis suggest that a decrease in the number of beneficial symbionts and an increase in the number of pathogens could be involved in the pathogenesis of several conditions. In PD, the oral microorganism composition could shifts from primarily of gram-positive aerobes to primarily of gram-negative anaerobes[30]. The development of oral dysbiosis is likely to occur over an extended period of time. During this period, the oral health of the host

deteriorates until a state of clinical disease occurs. Simultaneously, a succession of microbial complexes develops.

1.1.5. Pathogenesis

Page and Schroeder in a classic histopathology experiment have defined the development of the periodontal lesion in 4 different stages[31].

1.1.5.1. Initial lesion

It is an inflammatory response detectable after 2-4 days of plaque accumulation. It is characterized by vasodilatation, loss of perivascular collagen, margination and active migration of neutrophils and monocytes into the periodontal tissues and junctional epithelium mediated by Intercellular adhesion molecule (ICAM) and Endothelial leukocyte adhesion molecule (ELAM). A higher flow of GCF results from the capillaries vasodilation in attempt to remove toxins.

1.1.5.2. Early lesion

It is observed after 7-14 days of plaque build-up and corresponds to the gingivitis clinical picture. Neutrophils and lymphocytes represent the main immune response cells detectable below the basal cells of the junctional epithelium (Figure 3). This migration is facilitated by the breakdown of the gingival connective tissue. Matrix metalloproteinases (MMPs) derived from the biofilm and host response contribute to the reduction of the collagen fibers[32].

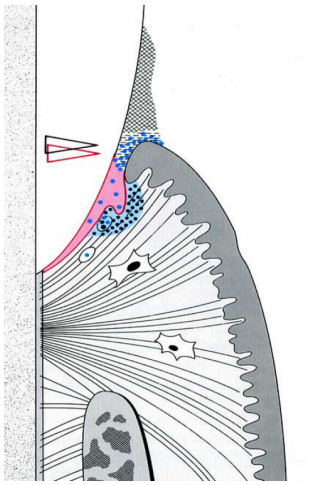


Figure 3 Early lesion

PMN and a widening of the sulcus form a barrier against bacteria. Lymphocytes infiltrate starts to appear.

Adapted from Rateitschak 2005

1.1.5.3. Established lesion

Histologically, the inflammatory infiltrate moves towards the oral epithelium and is represented mainly by plasma cells but in younger patients lymphocytes remain the main population detected[33]. There is an ulceration and hyperplasia of the junctional epithelium. Its clinical features are swelling and bleeding after probing.

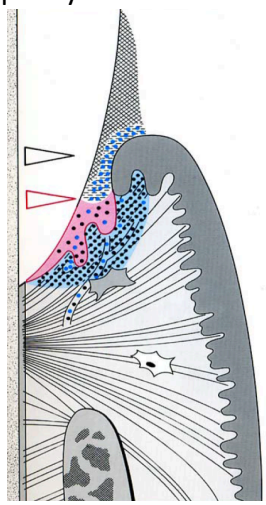


Figure 4 Established lesion

Plaque accumulation triggers the host response and the dislocation of the epithelium. Adapted from Rateitschak 2005

1.1.5.4. Advanced lesion

It involves the whole periodontium causing loss of tooth connective tissue attachment fibers. At this stage, the junctional epithelium apical migration modifies the sulcus anatomy leading to a periodontal pocket. The periodontal damage is mediated by an increased osteoclast activity, MMPs release and dysregulation of host derived factors such as proteinases and proteinase inhibitors, matrix metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs), pro-inflammatory cytokines such as Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), Tumour Necrosis Factor-α (TNF-α) and others, prostaglandins and the products of polymorphonuclear leukocytes[34].

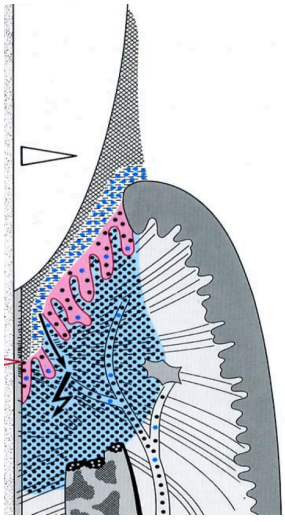


Figure 5 Advanced lesion

Loss of attachment, bone reabsorption and epithelial ulceration

Adapted from Rateitschak 2005

1.1.6. The host response

The host system reacts to the presence of non-self antigens with the innate and adaptive immune response. The innate mechanism is in place from birth and does not change in response to a specific antigen. The adaptive immunity instead is specific and it is developed during the lifetime[35]. Skin and mucous membranes are physical barriers against the invasion of infectious agents. Fluids such as saliva, tears, urine, GCF have also a clearing washing action. In addition they contain anti-bacterial molecules. The intact gingival epithelium is an obstacle to bacterial invasion and secretes substances toxic to various microorganisms. The saliva explicates a both a mechanical and chemical action flushing the oral cavity and providing agglutinins and antibodies. The GCF has the same function in the gingival sulcus. Furthermore the resident non-pathogenic bacterial community competes with harmful microorganisms for nutrients and attachment sites and produces substances to limit their colonization. Following physical damage of the host barrier with subsequent bacterial invasion, the innate immune response acts starting the inflammatory process, classically defined by the Latin terms dolor, rubor, calor and tumor. Its aim is to stop the bacterial invasion but it might have also detrimental effects such as the chronic inflammatory damage observed during periodontitis.

1.1.7. Risk factors

A risk factor can be defined as "an aspect of personal behavior or lifestyle, an environmental exposure, or an inborn or inherited characteristic, which on the basis of epidemiological evidence is known to be associated with a health related condition". Identifying a risk factor increases chance of a specific disease occurrence. Periodontitis is not purely the consequence of a microbial presence, multiple risk factors contribute to its development and progression[36-38].

1.1.7.1. Modifiable risk factors

1.1.7.1.1. Oral microorganisms

There are several subgingival microorganisms in periodontal lesions, however only a small number has been associated with the progression of the disease. Gram-negative anaerobic rods and spirochetes represent the majority of the species detected in deep lesions. Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans are related to the pathogenesis of periodontitis[39,40]. Furthermore, Bacteroides forsythus, Prevotella intermedia, Peptostreptococcusmicros and Fusobacteriumnucleatum seem to be linked with its progression[41].

1.1.7.1.2. Tobacco

Smoking represents a major risk factor for all cause mortality; it is related to both a higher rate of periodontitis progression and tooth loss. Cigarette smoke contains thousands of toxins, such as carbon monoxide, oxidizing radicals, carcinogens and nicotine. Strong evidence suggests that tobacco smoke has a detrimental effect on the periodontium[42]. First of all, cigarette smoking was found to select for specific periodontal pathogens, including P. gingivalis, Treponema denticola and T. forsythia, suggesting a shift of the oral microflora towards a more pathogenic one with an increased risk for the onset and progression of the disease[43]. Secondly, even at low doses, nicotine is linked to peripheral vasoconstriction and vascular dysfunction[44]. That accounts for the lower gingival bleeding and inflammation observed in smokers compared to non-smokers. The subsequent vasoconstriction could plausibly reduce the oxygen tension in the periodontal lesion favoring anaerobes species growth. Nicotine action can also alter neutrophils function including their proliferation and chemotaxis as well as affecting fibroblasts homeostasis. It is well known that tobacco

exposure not only increases odds for diagnosis of PD but also negatively impacts on the wound healing processes following periodontal treatment. Lastly current smokers show a higher level of pro-inflammatory cytokines and CD3, CD4 and CD8+ T-cell sub-populations, all related to greater periodontal tissues destruction[45].

1.1.7.1.3. Alcohol

Alcohol consumption, depending on the dosage, has been associated with the severity of periodontitis[46]. Evidence from observational studies suggests a linear relationship between number of alcohol units per week and clinical attachment loss[47]. However, more studies are required to elucidate the mechanisms by which alcohol affects the periodontium.

1.1.7.1.4. Medications

Using specific medication may interfere with the oral homeostasis. Various drugs including antihypertensive, narcotic analgesics, some tranquilizers and sedatives, antihistamines, and antimetabolites can decrease the salivary flow[48]. Furthermore anticonvulsants, calcium channel blocking agents, and cyclosporine may induce gingival overgrowth and predispose to more dental plaque accumulation and gingival inflammation[49].

1.1.7.1.5. Stress

Stress could increase the risk of developing periodontitis[50]. It has been associated with poor plaque control, higher serum levels of glucocorticoids that might affect the immune system and insulin resistance. Anger, financial issues and work stress have all been linked to the worsening of periodontal conditions[51].

1.1.7.1.6. Obesity

Obesity and overweight, assessed by body mass index, waist/hip ratio, waist

circumference, body weight, and body-weight changes, are a major public health problem. More than one-third of the adult population and about one-fifth of children and adolescents classified as obese. The accumulation of adipose tissue is linked to insulin resistance and chronic systemic inflammation. Being overweight is associated with increased risk of co-morbidities including diabetes, cardiovascular disease and cancer. Evidence indicates that obese individuals are more susceptible to infections due to an altered immune response caused by increased systemic inflammation [52].

Periodontitis has been repeatedly associated with adiposity, suggesting a potential role of adipose tissue in increasing local and systemic inflammation and subsequently the susceptibility to PD itself. A systematic review of observational studies (cross-sectional) confirmed an increased prevalence and severity of PD in obese and/or overweight individuals, with a doubled odds ratio for diagnosis of PD (OR=2.13, 95% confidence interval: 1.40–3.26)[53].

A number of mechanisms have been proposed to explain the association between obesity and PD including altered microflora (overgrowth of T. forsythia) and differences in saliva composition and immune responses. There is a body of evidence supporting this hypothesis however further studies are required to enlighten the mechanisms underlying this link.

1.1.7.2. Non-modifiable risk factors

1.1.7.2.1. Age

The prevalence and severity of periodontitis increase with age. The mean annual bone loss rate in 70 years old population is 0.28 mm compared to 0.07 mm in 25 years old individuals[54]. This could be explained with the longer period of time in which the periodontal tissues have been exposed to the bacterial challenge. However

observational studies have reported that in elder populations only a small proportion of individuals is affected by severe bone loss. On the other hand, advanced periodontal destruction and bone loss are seldom seen in individuals under the age of 40. Therefore it is not clear if age leads to increased susceptibility or that there are cumulative factors that control the onset of periodontitis.

1.1.7.2.2. Gender

The male sex is the most prevalent risk factor for periodontal disease[55,56]. However, there does not seem to be any gender related genetic difference in the susceptibility to periodontitis. It seems that the male populations has a higher level of periodontal destruction possibly related to the lower oral health awareness. Men show higher level of disease in terms of prevalence, extent and severity. Indeed, male patients had about 50% higher prevalence of periodontitis; had 33% more mild, 28% more moderate and 180% more severe periodontitis when compared to females[57].

1.1.7.2.3. Genetic factors

Specific genotypes are significantly associated with the severity of periodontal diseases[58]. Severe periodontitis affects only around 10% of the population[15]. Studies investigating familial aggregation, report that aggressive PD is an inherited trait with up to 50% of siblings being affected[59]. There is a lower number of studies looking at the familial aggregation in chronic periodontitis and it is controversial the relevance of genetic compared to other environmental factors[60].

1.1.7.2.4. Diabetes

Diabetes mellitus (DM) is a group of diseases characterized by hyperglycemia due to the alteration of the glucose metabolism regulated by the hormone insulin[61]. It represents a global matter affecting more than 8% of the population only in the USA[62].

It is associated with increased mortality and its main complications, such as cardiovascular and renal diseases are responsible for a high level of morbidity[63]. Periodontitis and diabetes are both common chronic conditions which often co-exist in the same patients. Patients with diabetes often present with poor oral health and increased prevalence of periodontitis [64]. In addition, periodontal infection could worsen the glycemic control defining a potential bidirectional relationship between these two diseases [65]. Numerous large observational studies on the Pima Indians population reported that the severity of PD is higher in patients with uncontrolled DM[66]. DM could affect the periodontium by a number of mechanisms mainly linked to up-regulation of local and systemic inflammatory response. Patients with PD and DM exhibit higher levels of pro-inflammatory mediators in the GCF and increased monocytes production of pro-inflammatory cytokines when compared to patients with PD without DM. Hyperglycemia is a recognized trigger of inflammation, oxidative stress, apoptosis, and periodontal tissue destruction. The inflammatory nature of both DM and PD hence could be responsible for their two-way relationship.

1.1.7.2.5. Osteoporosis

Postmenopausal osteoporosis may be related to dental osteopenia, specifically in the mandible[67]. Evidence from different reviews suggest that osteoporosis has a role in the expression of periodontal diseases showing a direct association between skeletal and mandibular osteopenia and loss of alveolar crestal height and tooth loss in postmenopausal women[68]. However there is a difference between osteopenia, postmenopausal osteoporosis and osteoporosis. Periodontitis and osteopenia may have common etiological agents that may either directly influence or modulate both disease processes.

1.1.8. Treatment of periodontitis

The main goal of periodontal treatment is to guarantee an adequate infection control reducing the microbial burden to a non-clinical detectable level. This is achieved with a long term structured treatment plan that is divided in different stages. Firstly, providing oral hygiene instructions to each patient in order to reach an optimal supra-gingival plaque control and removing any retentive factor represented by faulty restorations. Secondly, accessing the periodontal lesions removing any sub-gingival debris and bacterial biofilms from the root surface of the affected dentition by means of non-surgical instrumentation. Thirdly, correcting any anatomical unfavorable bone anatomy by a surgical approach. Lastly, reducing the rate of recurrences with a regular monitoring and supportive periodontal treatment.

1.1.8.1. Non-surgical periodontal therapy

Evidence from different systematic reviews reports a consensus on the efficacy of supra and sub-gingival debridement in the reduction of the periodontal lesions and improvement of clinical attachment level[3,69]. The instrumentation of the affected dentition, scaling and root planing (SRP) with ultrasonic or hand instruments, in association with optimal daily oral hygiene measures is aimed to alter the microbial biofilm and reduce the host local inflammatory response. The non-surgical treatment can be delivered either divided per jaw quadrant in different sessions or in a full-mouth manner in order to prevent the re-infection of the treated sites[70]. There does not seem to be any difference in the clinical outcome of these two non-surgical approaches[71].

1.1.8.2. Surgical periodontal therapy

Nonsurgical periodontal treatment leads to an improvement in clinical attachment levels and a reduction/elimination of the inflammation. However, surgical therapy is indicated in specific clinical situation in which there is a persistence of the periodontal lesion to adequately debride the root surface, root concavities and furcations and recreate a more favorable anatomy for gingival tissues[70].

1.1.8.3. Supportive periodontal therapy

It is well accepted that regular maintenance care is essential for the long-term success of periodontal therapies. Supportive periodontal therapy (SPT), formerly referred to as periodontal maintenance include an update of the medical and dental histories, examination of extra- and intraoral soft tissues, dental examination, evaluation of the patient's oral hygiene performance and supra- and sub-gingival removal of bacterial plaque and calculus. The therapeutic goals of SPT are to: i) prevent or minimize the recurrence and progression of periodontal disease , ii) prevent or reduce the incidence of tooth loss.

1.2. Atherosclerosis

Atherosclerosis is a chronic condition that affects the surfaces of the medium to large size arteries consisting of the thickening of the vascular wall due the accumulation of lipids and fibrous components forming atherosclerotic plaques[72]. It potentially leads to ischemia of the tissues located distally to the lesions due to either stenosis of the blood vessel or, in the majority of the cases thrombus formation caused by the rupture of an atherosclerotic plaque[73]. According to the location of the artery occlusion, the subsequent ischemia may induce stroke, myocardial infarction or peripheral artery disease. Atherogenesis occurs already in childhood but its clinical manifestations are normally observed starting from the middle age[74]. Coronary heart disease and stroke, mainly due to atherosclerosis, are responsible for the majority of the death worldwide. This is why identification of new therapeutic strategies for its management has become a public health priority[75].

1.2.1. Arteries anatomy

Arteries are composite muscular, fibro-elastic vessels that function to transport blood to the body from the heart to the peripheries at high pressure[76]. Depending on the dimensions, they can be divided in large or elastic arteries, medium or muscular and arterioles. The arterial wall consists of 3 layers, tunicae that, according to the vessel size and vessel bed, have a different structure (Figure 6).

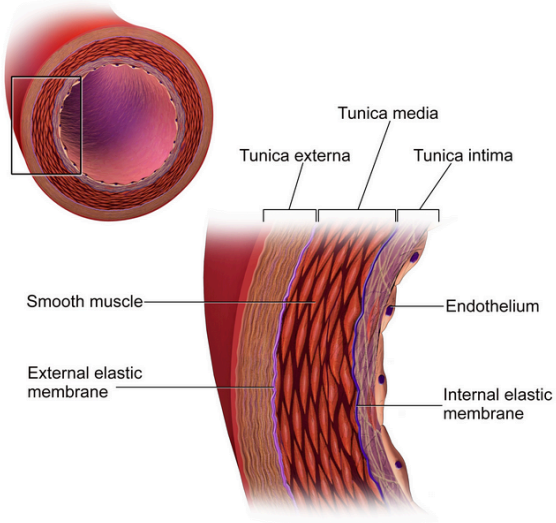


Figure 6 The structure of an artery wall

1.2.1.1. Tunica Intima

The inner layer of the artery is represented by the tunica intima, a narrow region separating the bloodstream from an elastic layer, the internal elastic lamina (IEL)[77]. In conduit arteries, there is also an underlying stroma containing multiple layers of vascular smooth muscle cells (VSMCs). The intima consists of a single layer of endothelial cells forming an important paracrine organ; the endothelium.

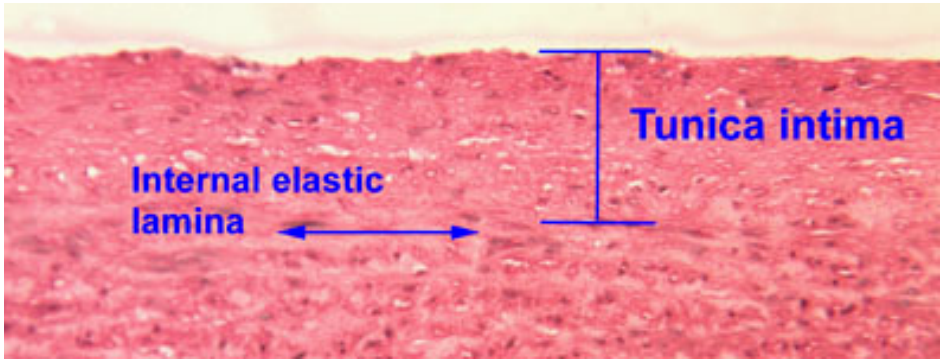


Figure 7 Tunica Intima

1.2.1.1.1. The endothelium

It forms the inner layer of the human vascular tree and is directly involved in atherogenesis since its functions can be directly affected by many inflammatory stimuli. It can cover an area of approximately $7m^2$ including 10^{13}cells[78]. The endothelium lies on a basement membrane surrounded by smooth muscle and connective tissue according to the vessel function and location. Endothelial cells are responsible for many vital functions[79,80].

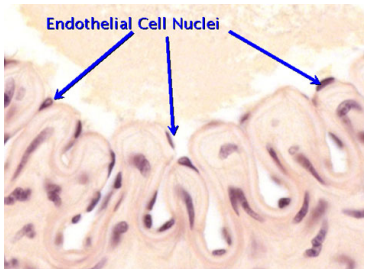


Figure 8 Endothelial cells

1.2.1.1.1.1. Regulation of the vessel tone

The endothelium can regulate the vessel tone in response to either chemical or mechanical stimuli releasing different vasoactive mediators including prostacyclin, nitric oxide (NO), hydrogen peroxide (H_2O_2) to relax the surrounding VSMCs or

thromboxane, endothelin or angiotensin II to contract it[81]. In addition, the endothelium, through the myoendothelial gap junctions, can communicate directly with the VSMCs releasing small calcium (Ca_2^+) ions causing its contraction[82].

1.2.1.1.1.2. Exchange of fluids and solutes

The passage of fluids and large molecules between the blood and the interstitial space is subject to regulation by the endothelial cells. The inter-endothelial gap junctions allow the exchange of small molecules but large solutes require a vesicular transport[79]. The inflammatory process can cause disruption of these mechanisms leading to edema.

1.2.1.1.1.3. Hemostasis

Hemostasis is the process by which the blood loss deriving from vessel wall damage is stopped. It requires vasoconstriction, platelets adhesion and activation at the site of the injury and the formation of a fibrin plug that seals the vessel. The endothelium, in normal conditions does not initiate the hemostasis however, during an inflammatory process, it can produce a specific molecule (tissue factor, TF) which in turn activates the clotting cascade. The endothelial cells produce pro coagulant mediators such as von Willebrand factor (vWF), plasminogen activator inhibitor-1 (PAI-1) or anticoagulant molecules including NO and prostacyclin[83].

1.2.1.1.1.4. Angiogenesis and vasculogenesis

The cell turnover, at a daily rate of approximately 0.1%, guarantees the integrity of the endothelium preventing a long-term dysfunction. The angiogenesis has been the first mechanism to be identified to repair any endothelial damage. Pre-existing cells duplication and migration from a vessel could allow growth, remodeling and healing of the endothelium[84]. An additional mechanism called vasculogenesis, could be

responsible for endothelium homeostasis. Cells originated in the bone marrow could be converted into new endothelial cells. Endothelial progenitor cells (EPC) have been identified and characterized as regulators of endothelium repair in various districts, however their exact role is still a matter of investigation[85].

1.2.1.1.1.5. Response to inflammation

The endothelial response to a pathological process is based on the production of inflammatory mediators and adhesion molecules to increase the leukocyte recruitment[86]. In physiological conditions, these pathways are localized and reversible, however a perpetuated inflammatory response is related to endothelial dysfunction and may be the first step towards atherosclerosis and CVD. The endothelium can release cytokines and chemokines in response to numerous *in vitro or vivo* inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), shear stress, infection or hypoxia. Cytokines such interleukin-1 (IL-1) and interleukin-6 (IL-6) can trigger fever, production of acute-phase reactants and activation of lymphocytes. Chemokines such as Interleukin-8 (IL-8) and monocyte chemoattractant protein (MCP-1) can recruit and activate neutrophils, monocytes, T cells, dendritic and natural killer (NK) cells. The up-regulation of adhesion molecules on the vessel wall determines a higher interaction between leukocytes and the vessel wall increasing their recruitment. P and E selectins adhesion molecules, expressed by the endothelium during the inflammatory response, facilitate the rolling of leukocytes on the endothelial surface. The chemokines activate the white cells causing an up-regulation of their surface integrins responsible for the interaction with endothelial intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) to engage the vascular wall[87].

1.2.1.2. Tunica media

It represents the middle and thickest coat of the arterial wall. It is contained by the internal and external elastic lamina. Multiple layers of low proliferative rates of VSMCs, together with long-lived elastic and more rapid turnover of collagen fibers, compose the tunica media. The media layer, except from cases of pathological thickening, is normally avascular. VSMCs with their contraction regulate the vessel diameter and also maintain the extracellular matrix (ECM).

1.2.1.3. Intima-media thickness

With aging and exposure to a variety of risk factors (smoking, obesity, increased blood pressure) smooth muscle cells and extra cellular matrix slowly accumulate within the intima layer, increasing its thickness and eventually developing atherosclerotic lesions. The development of high-resolution B-mode ultrasound technique has made possible to non-invasively study arteries and their structure. However, the thickness of the intima alone is below the resolution of the ultrasound equipment, and therefore not possible to measure. The combined intima plus media complex, is possible to measure and therefore an increased intima and media complex thickness (IMT) is used as an early sign of atherosclerosis[88,89].

1.2.1.4. Tunica Adventitia

Outside the EEL lies the adventitia, or outer layer of the artery, consisting mainly of collagen and elastic tissue. The adventitia provides nutrition to the vessel wall via the vasa vasorum, as well as innervation of the artery.

1.2.2. Epidemiology

Precursor lesions of atherosclerosis (intima-media thickening) may occur during adolescence, but the incidence of definite atherosclerotic lesions remains low until

middle age in men and the onset of menopause in women. Within the 5-year period following menopause the incidence rates of women are similar to those observed in men of equal age. However, in some subjects, the disease can progresses rapidly during their third decade of life. It has been estimated that in USA alone, 12.6 million people suffer from CHD and 4.6 million people from stroke[90]. Great Britain and in Scandinavia report the highest rate[91] of atherosclerotic manifestation, particularly in Scotland and Finland. In the past twenty years, the incidence rates have been decreasing in several industrialized countries but not in the developing world. This most likely is due to the public health initiative increasing prevention of atherogenesis via management of common risk factors: serum cholesterol concentration, hypertension and smoking[91].

1.2.3. Risk Factors

Current major cardiovascular risk factors are summarized in Table 4. Seminal epidemiological work, such as the Framingham Study[92] helped identify the classical risk factors for CVD including: male sex, increasing age, family history, smoking habit, presence of diabetes, obesity (especially high levels of visceral adiposity), hypertension, hyperlipidemia and a sedentary lifestyle

Table 4 Classic risk factors for CVD
Non-Modifiable: gender, age, familiarity
Modifiable: Smoking, blood pressure, diabetes, sedentary lifestyle, adiposity, high cholesterol

Most of these risk factors interrelate in some way either directly or indirectly. Excluding the non-modifiable factors including age, sex and family history, management of atherosclerosis is achieved by controlling its main risk factors such as smoking, diabetes, hyperlipidemia, adiposity, and blood pressure. These established risk factors are believed to account for somewhere in the region of 70-90% of incident CHD cases. Emerging CVD risk factors nevertheless have been identified (summarized in Table 5) and fall into numerous overlapping categories.

Table 5 Emerging potential risk markers for CVD
Metabolic/ Dietary markers: Triglycerides, impaired glucose tolerance, metabolic syndrome, leptin, adiponectin
Novel lipids: LP(a), apoA, apoB
Inflammatory markers: Fibrinogen, PV, WCC, hsCRP, IL-6, TNFα, IL-18, sCD40L, MMPs, α oxLDL, Abs
Markers of endothelial activation/damage: oxLDL, sICAM-1, NO, brachial artery reactivity, glutathione dysfunction
Thrombotic markers: t-PA, PAI-1, D-dimer, vWF, homocysteine
Non-invasive imaging biomarkers: Ankle brachial pressure index (ABI), MRI angiography, carotid IMT, CT coronary calcification
Invasive imaging biomarkers: Intravascular ultrasound, coronary angiography

1.2.4. Pathogenesis

Histologically atherogenesis starts with the formation of fatty streaks that are small sub-endothelial deposits of lipids. Subsequently, as part of an inflammatory response, monocytes are recruited from the bloodstream to enter the artery wall differentiating

into macrophages, and then slowly turn into large foam cells. The initial lesion appears as a fatty streak, it is initially composed by lipid-laden monocytes and macrophages (foam cells), together with T-lymphocytes within the arterial wall. Subsequently, smooth muscle cells migrate towards the fatty streaks, T-cell are activated by tumor necrosis factor (TNF-α), interleukin-2 (IL-2), and granulocyte–macrophage colony stimulating factor. Upon stimulation from oxidized low-density lipoprotein, macrophage colony-stimulating factor, TNF-α, and interleukin- 1 (IL-1), recruited macrophages develop into foam cells. In response to an injury, a fibrous healing response is observed. Finally, platelets adhere and aggregate to the plaque subsequent to endothelial injury . This is the result of stimulation from integrins, P-selectin, fibrin, thromboxane A2, and tissue factor, and is accountable for leukocyte adherence. The lesions develop from an intermediate stage to an advanced level in association with fibrous cap formation near to the walls of the lesion. The fibrous cap covers a mixture of leukocytes, lipids, and debris, which can cause the formation of a necrotic core. The continuous adhesion and entry by leukocytes causes lesions to expand at their shoulders. Macrophage colony-stimulating factor, monocyte chemotactic protein-1 (MCP-1), and oxidized low-density lipoprotein are principle factors associated with macrophage accumulation. The result of apoptosis and necrosis, increased proteolytic activity, and lipid accumulation is the necrotic core. A number of contributing factors are associated with the formation of the fibrous cap. These include increased activity of platelet-derived growth factor, transforming growth factor ß, IL-1, TNF-α and osteopontin. The rupturing or ulceration of fibrous caps usually occurs in thinning regions that cover advancing lesions. The fibrous cap starts to thin because there is a continuous influx and activation of macrophages. The macrophages at these sites release matrix-metalloproteinases (MMPs) and other proteolytic enzymes that act to

degrade the matrix. This can then ultimately lead to incidences of haemorrhages from the vasa vasorum or from the lumen, and can cause the formation of a thrombus and occlusion of the artery.

1.2.5. Prevention and Treatment of atherosclerosis

The prevention of atherosclerosis represents a long-term effort to all health care professionals and the basis of many public health policies. The aim of both individual practitioners and organizations providing health care is to optimize the risk factor profiles before clinical atherosclerotic disease becomes clinically evident.

1.2.5.1. Lifestyle intervention

The ACC/AHA 2013 Guideline on Lifestyle Management to Reduce Cardiovascular Risk suggests lifestyle interventions evaluated in randomized clinical trial relying primarily on biomarkers or surrogate endpoints rather than "hard" cardiovascular outcomes[93]. The health risks related to tobacco should be provided encouraging smoking cessation. Similarly, prudent dietary and physical activity habits for maintaining ideal body weight should be recommended. Both National Institutes of Health (NIH) and AHA statements recommend at least 30 min of moderate-intensity physical activity per day. Obesity, specifically the male pattern of centripetal or visceral fat accumulation, can contribute to the onset of metabolic syndromes.

1.2.5.2. Lipid-lowering intervention

Statins are medications administered to decrease the low-density lipoprotein (LDL) cholesterol and multiple trials have reported their benefit on the CV mortality and on the risk of major cardiovascular events[94]. The linear relationship between LDL-cholesterol and cardiovascular risk suggested that statins main benefit was related only to the reduction of LDL-cholesterol. However, a number of additional effects of

statins have been suggested to contribute to their efficacy in CVD. The Heart Protection Study reported that simvastatin reduced mortality and morbidity even in patients with 'normal' LDL-cholesterol levels[95]. Their potential benefit could also be explicated by improvements in endothelial function, halting or retardation of atheroma development, reduction in inflammation and antithrombotic effects[96].

1.2.5.3. Anti-Platelet intervention

The potential benefit of the anti-platelet therapy is related to the reduction of thrombus formation and vascular inflammation[97]. Aspirin has been widely experimented for secondary prevention in patients with established atherosclerotic vascular disease. In addition, the combination of statin and aspirin are associated with the greatest reduction in mortality in a case-control analysis. Furthermore, other antiplatelet agents such as clopidogrel, prasugrel and ticagrelor have been introduced in the management of atherosclerosis manifestation showing encouraging results[98].

1.2.5.4. Anti-coagulation intervention

The coagulation cascade involves an interaction between the contact activation and the tissue factor pathway leading to the conversion of factor X to Xa, initiating the common pathway[99]. The subsequent conversion of pro-thrombin to thrombin, catalyzing the formation of fibrin, leads to the formation of a firm clot stabilized by aggregated platelets[100]. Initially, vitamin K antagonists, such as warfarin, were the only anticoagulant treatment widely available for human use. Approximately 65,000 patients are treated in US emergency departments annually for anticoagulants-related hemorrhagic events[101]. The high rate of adverse events requesting a strict monitoring of the patients stimulated the research of potentially safer medications. Targeting different steps of the coagulation process allowed the introduction of multiple novel

anticoagulants such as direct thrombin inhibitors (e.g. dabigatran), and factor Xa inhibitors (e.g. rivaroxaban, apixaban)[102].

1.2.5.5. Anti-hypertension intervention

Evidence from several RCTs report anti-hypertension medications, such as β-blockers, as effective in the reduction of recurrent acute myocardial infarction (AMI), sudden cardiac death and total mortality in patients with AMI[103]. Their beneficial effect is related to the reduction of the heart rate and blood velocity with a consequent lower flow turbulence and vascular wall stress. In addition, recent analyses support a positive effect of β-blockers on the progression of atherosclerosis[104]. Furthermore, renin-angiotensin system inhibition improves the endothelial function and RCTs show a reduction in coronary events not only related to a blood pressure reduction suggesting a stabilizing effect on the atheroma[105].

1.2.5.6. Anti-inflammatory intervention

Evidence of an immune activation and cytokine signalling within atherosclerotic lesions support the inflammatory pathogenesis of atherosclerosis[106] supporting the role of inflammatory biomarkers as independent risk factors for acute CV events[107] and the involvement of LDL particles and their contents to activate innate and adaptive immunity[108]. Furthermore, animal models of atherosclerosis targeting the disruption of cholesterol-regulating genes leading to atherosclerosis report that interfering with immune signalling and inflammatory mediators has an anti-atherogenic effect[109-111]. Therefore, this evidence has encouraged the development of anti-inflammatory strategies for prevention and treatment of atherosclerosis targeting the inflammatory signalling cascades, pro-inflammatory cytokines, and inflammatory autacoids.

1.3. Diabetes

Diabetes mellitus is a group of diseases characterized by hyperglycemia resulting from a deficiency in the body's ability to produce insulin or due to a resistance to its action[112]. Diabetes is associated with a higher risk of vascular damage observable as micro-vascular complications such as retinopathy, nephropathy and neuropathy, or macro-vascular complications including ischemic heart disease, stroke and peripheral vascular disease[113]. As a consequence, diabetes is linked to a deteriorated life quality and expectancy.

1.3.1. Diagnosis

The diagnosis of diabetes is based on the glycemic level above which there is an increase of micro-vascular complications. The World Health Organization (WHO) has adopted the diagnosis criteria introduced in 1997 by the American Diabetes Association[114,115]. The diagnosis of diabetes can rely on three different criteria (Table 6)

Table 6 Diabetes diagnosis criteria
Random plasma glucose concentration ≥ 200 mg/dl (≥ 11.1 mmol/l) and classic symptoms of diabetes such as polyuria, polydipsia, and unexplained weight loss
fasting plasma glucose ≥126 mg/dl (≥7.0 mmol/l)
2-h post-load glucose ≥200 mg/dl (≥11.1 mmol/l) during an oral glucose tolerance test

The diagnosis requires the confirmation on a different day of any of these criteria. With regards to the impaired glucose tolerance, it is based on the oral glucose tolerance test; a 2-h post-load plasma glucose concentration ≥140 mg/dl but ≤199

mg/dl (between 7.8 and 11.1 mmol/l) allow its diagnosis. Meanwhile, a fasting plasma glucose ≥100 mg/dl but ≤125 mg/dl (between 5.6 and 6.9 mmol/l) define an impaired fasting glucose. The hemoglobin A1c test is used to monitor the overall glycemic control in subjects with diagnosed diabetes.

1.3.2. Classification

WHO released the first widely recognized classification in 1980 including two major categories of diabetes mellitus[116]:

- Insulin-dependent diabetes mellitus (IDDM)
- Non insulin-dependent diabetes mellitus (NIDDM)

and other forms such as gestational diabetes. In 1985 it was suggested to use the terms IDDM and NIDDM proposing to classify the patients according to their treatment rather than following the pathogenesis[117]. The terms Type I and Type II were introduced to describe a form related to pancreatic islet beta-cell destruction for the former and diabetes resulting from defects in insulin secretion for the latter. In 1997, the American Diabetes Association released a new classification that was then modified in 2003[112].

1.3.2.1. Type I Diabetes (T1DM)

T1DM is the consequence of the destruction of the pancreatic β-cells by an immune-mediated process that leads to the lack of insulin secretion. The higher incidence is observed during adolescence but approximately 15-30% of the cases can occur after 30 years of age[118]. The alteration in the glucose metabolism due to the reduction or absence of insulin secretion requires the administration of exogenous insulin for the patient survival to avoid ketoacidosis, a potentially deadly condition. Specific antibodies for the pancreatic cells are detectable in T1DM patients[119] and monozygous

twins have a concordance for T1DM of 30–50%.

1.3.2.1.1. Idiopathic diabetes

In a minority of T1DM cases, ketoacidosis can occur with no evidence of autoimmune destruction processes or insulopenia. This form of diabetes seems to be detectable in African and Asian heritage and has a strong association with a positive familiar history. The insulin therapy requires periodic adjustment according to the state of the disease.

1.3.2.2. Type 2 Diabetes (T2DM)

It is the most common form of diabetes. It is preceded in the majority of the cases, by a pre-diabetic state in which glycaemia is abnormal but not reaching the level required for being diagnosed as diabetes. Some long-term damage to the body, especially the heart and circulatory system, may already be occurring during pre-diabetes. The pathogenesis of T2DM related to either to lower levels of insulin production or the dysfunction in its absorbance[120].

1.3.2.3. Gestational diabetes

This class of diabetes is detected for the first time during pregnancy. It is associated to overweight and family history of diabetes. If untreated, it can cause complication to the newborn. In addition, it is linked to a higher risk of developing T2DM for both the mother and the infant[121].

1.3.3. Epidemiology

The Caucasian heritage has the higher risk of developing T1DM. Starting form 1950 there has been a linear increase of T1DM cases in Scandinavia, the UK, and the U.S.[122]. Potential triggers explaining the pancreatic cells destruction could be the autoimmune response to cow's milk protein [123] or an enterovirus infection[124]. Scandinavia has the highest T1DM incidence with more than 30 cases/year/100,000 people meanwhile

Asia has the lowest number of new cases with 0.5 cases/year/100,000[125]. The prevalence of T2DM is estimated, only in the U.S., to be approximately 16 million people and an additional 30–40 million with impaired glucose tolerance[126]. Native Americans seem to be the most affected population.

1.3.4. Risk factors

Diabetes is associated with both modifiable, such as obesity and sedentary life-style, and non-modifiable, including ethnicity and genetics, risk factors. In T1DM the family history represent the major contributor, therefore the American Diabetes Association recommends that anyone with a first-degree relative with T1DM should be screened for diabetes. An important risk factor is also a pancreatic damage deriving from a trauma or a disease. For T2DM obesity, diet, physical inactivity, increasing age, insulin resistance, family history of diabetes, genetic factors, and race and ethnicity are all recognized risk factors. Specific genetic variations are related to its onset and certain ethnic groups such as African Americans, Mexican Americans, American Indians, native Hawaiians and some Asian Americans have a higher incidence of T2DM with African Americans as the highest risk population. Dietary regime and inactivity are contributing to its faster development in highly developed Countries.

1.3.5. Pathogenesis

Glucose deriving from the digestive system reaches the bloodstream elevating blood glucose levels. The hyperglycemia stimulates the secretion of insulin from the beta cells of the pancreas to allow cell glucose intake. Insulin binds to specific cellular receptors and facilitates entry of glucose into the cell, which uses the glucose for energy. The increased insulin secretion from the pancreas and the subsequent cellular utilization of glucose results in lowering of blood glucose levels. In diabetes, the insulin

production and secretion can be altered causing a change in the glucose metabolism. Similarly, a lack of the insulin function at a cellular level may also affect the glycaemia[127].

1.3.6. Treatment of Diabetes

Diabetes is considered the sixth leading cause of death by disease in the U.S. Its treatment as well as the management of diabetes-related complications is a priority for Governments worldwide, since the economic burden in 2007 alone exceeded $174 billion (Dall et al, 2007).

1.3.6.1. Lifestyle intervention

1.3.6.1.1. Diet

The diet adjustment is a fundamental tool in the treatment of diabetes. It aims to reduce the weight and to regulate the glucose and lipid daily absorption[128] recommending that carbohydrates should represents approximately 60% of the calories intake[129]. Lipids should not pass 30% and proteins approximately 20% of the diet. The alcohol consumption should be of one drink for adult women and two drinks for adult men and with meals.

1.3.6.1.2. Physical activity

A regular physical exercise has beneficial effects since it favors weight loss and improves the cardiovascular fitness. Moderate intensity activity can decrease the glycaemia, fasting and post-prandial insulin concentrations and increase insulin sensitivity[130]. With regards to the CV complication, regular exercise can lower the lipid profile and the blood pressure.

1.3.6.2. Pharmacological intervention

The control of blood glucose profile is related to a lower incidence of diabetic complications therefore, the reduction of hyperglycemia represents the main purpose of the diabetic therapy[131].

1.3.6.2.1. Insulin therapy

All the patients with T1DM, T2DM and gestational diabetes patients in whom other measures are not sufficient to control the glycaemia require insulin therapy. It is provided via subcutaneous injection. Its main side effects are increased risk of hypoglycemia and weight gain[131]. The insulin formulation available can differ in their pharmacological properties[132] with the aim of reduce the risk of hypoglycemia. It normally consists of a bolus dose given subcutaneously with a syringe. Otherwise insulin pump or continuous subcutaneous insulin infusion therapy is used as alternative.

1.3.6.2.2. Oral medications

1.3.6.2.2.1. Sulfonylureas

They promote the secretion of insulin binding to a specific receptor of the pancreatic cells[133]. The majority of the patients taking sulfonylurea will require combined therapy to achieve a good glycemic control since the drug alone does not reach the target. The side effects related are weight gain and hypoglycemia.

1.3.6.2.2.2. Meglitinides

The meglitinides target is an adenosine triphosphatase-dependent potassium channel; their action requires the presence of glucose[134]. Due to their rapid onset and short duration, they are given before meals. Meglitinides can be quite effective in the glycemic control and their side effects include weight gain and hypoglycemia, although

the risk of hypoglycemia is considerably lower than with sulfonylureas.

1.3.6.2.2.3. Biguanides

Metformin, a second-generation biguanide, is a common glucose-lowering agent acting on the inhibition of the liver gluconeogenesis and the reduction of the insulin resistance[135]. Metformin reduced the incidence of T2DM by 31% in individuals with impaired glucose tolerance or impaired fasting glucose[130]. It is effective as a mono-therapy, not associated with weight gain and the risk of hypoglycemia is low. It is contraindicated, however in renal dysfunction, hepatic dysfunction, hypoxemic conditions, severe infection and alcohol abuse.

1.3.6.2.2.4. Thiazolidinediones

Their action increase the insulin sensitivity[136] therefore they are particularly indicated in cases of insulin resistance. Adverse effects include increase in body weight, fluid retention, and plasma volume expansion, slight decreases in the hemoglobin level and rarely hypoglycemia. The risk of hepatotoxicity related to their use requires periodic measurements of transaminases.

1.3.6.2.2.5. α-Glucosidase inhibitors

They lower the glucose entry in the bloodstream inhibiting the breakdown of carbohydrates by specific enzymes in the small intestine. The delay in their digestion to distal part of the small intestine reduces the velocity of glucose entry in the circulation[137]. Potential side effects are bloating, abdominal discomfort, diarrhea, and flatulence.

1.4. Association between Periodontitis and Systemic Diseases

1.4.1. Introduction

The concept of focal infection has been very popular in medicine starting from the end of XIX to the first half of XX century. Microorganisms disseminated from the oral cavity were believed to provoke a wide range of systemic conditions for which the pathogenesis was still unclear[138]. As a consequence preventive teeth extractions was considered a beneficial treatment for the primary or secondary prevention of multiple conditions affecting the heart, liver, pancreas and kidneys. However, the advancement of medical research and the lack of a clinical efficacy of these measures led, by 1950, to the decline of this hypothesis. Since the last decade of the XX century, a new research trend investigating the potential role of periodontitis as a contributor to systemic disease has flourished[139]. Numerous evidences link PD to several systemic diseases including cardiovascular diseases, diabetes, pre-term birth and rheumatoid arthritis. Results from observational studies are continuously introducing new possible conditions associated with periodontal infections[140].

1.4.2. Periodontitis and Cardiovascular Diseases

Multiple microorganisms have been linked to the atheroma; for instance, Chlamydia pneumonia, a bacterium responsible for respiratory tract infections, have been isolated from the arteries of CV patients[141] and detected in higher levels in patients with CHD compared to controls[142]. The first observational study linking poor oral health to CVD analyzed 211 patients who suffered from a MI comparing their oral health status to 366 controls. The investigators reported a lower level of periodontal health in the test group[143]. Subsequently, epidemiological evidence from multiple observational trials has linked PD to cardiovascular events[144-154]. However not all

observational data supports this association[155-157]. The majority of the trials have been conducted in US with up to 23 years as a follow-up period subsequent to the periodontal examination. The assessment of periodontitis varied between the studies ranging from radiographic assessment of the bone loss, total dental index, Russel's periodontal index and surveys. Cardiovascular events represented by MI, stroke, hospitalization for CHD were considered as outcomes. The potential confounders adopted for the adjustment of the results were classic risk factors for CVD. The estimates of the association reported a relative risk (RR) from 1.2 to 7.0[158]. However, the robustness of these data could be undermined by an incomplete adjustment for confounders. Data from the first National Health and Nutrition Examination Survey (NHANES I) suggested the absence of a moderate association between PD and CVD but could not rule out a small magnitude causal association[156]. The main limitations of the NHANES-I were related to the assessment of PD, the Russel's Index, which could have led to an underestimation of exposure to PD and reduced the strength of the link. In addition, there were no measures with regards to possible causal mechanisms and also the exposure was only assessed at baseline [154]. A systematic review of 31 observational studies identified a positive link between PD and CVD, stroke or peripheral vascular disease[158]. Furthermore, a meta-analysis of observational studies assessing the relationship between PD and total CHD/CVD reported that patients with periodontitis had an overall adjusted future risk of CHD 1.15 times (95% confidence interval [CI]: 1.06 to 1.25; P = 0.001) higher than otherwise healthy individuals. The RR calculated for future CVD was 1.17 (95% confidence interval [CI]: 1.03 to 1.34; P = 0.001)[159]. This association has been systematically reviewed several times during the last decade[160-163] and recently, the American Heart Association working group concluded that PD is associated with atherosclerosis independently of known

confounders[164]. Furthermore the consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases concluded that robust epidemiologic evidence support periodontitis as a contributor to an increased risk for future cardiovascular disease[165].

1.4.3. Periodontitis and Diabetes

Strong evidence link DM, a group of metabolic disorders[114], to the prevalence and severity of gingival inflammation and PD suggesting the glycemic control as a potential determinant of this association[166,167]. Adolescent affected by Type 1 diabetes present a higher level of periodontal inflammation affecting approximately double of the sites compared to controls with the same amount of plaque[168,169]. In addition, in the same population, poor glycemic control is associated with the severity of the oral inflammation[170] showing a negative correlation between level of glycaemia and gingivitis[171,172]. 263 patients with type 1 diabetes compared to 59 relatives without diabetes and 149 unrelated controls were screened for PD. 13.6% of patients with diabetes had PD between the age of 13 and 18 years with an increased prevalence of 39% among those aged 19 to 32 years. The prevalence in controls without diabetes was lower than 3%[168]. The same correlation has been reported in adults with type 1 diabetes[173] and data from a longitudinal study on experimental gingivitis suggest that, with the same level of plaque control, adults with Type 1 diabetes develop gingival inflammation more rapidly and with a higher severity[174] than controls.

Type 2 DM has been strongly linked to PD [175]. Observational studies report a higher extent and severity of PD in T2DM[66,176-178]. A meta-analysis of four studies[177,179-181] including 3.524 adults with diabetes reports that the overall estimated correlation coefficient was 0.19 (95% confidence limits 0.16 to 0.22)[166]. Pima Indians, a Native American of Arizona population with the world highest prevalence of T2DM, present

worse periodontal health in patients with diabetes in all age groups[66,177]. In addition, analysing a sample of 2.273 patients, PD was detected in 60% of the Pima Indians with diabetes meanwhile its prevalence was 36% in absence of diabetes[181]. A subset of 701 individuals with no signs of PD observed for an average of 2.5 years, presented with an incidence of PD 2.6-fold higher in patients who also had diabetes [181]. Further observational data from a cohort study with 2 years follow-up study, suggested a 4-fold risk of bone loss in T2DM patients when compared to controls[182]. The onset and progression of PD in patients with diabetes is related to the metabolic control. Established diabetic complications such as, retinopathy or nephropathy are detected in poorly controlled subjects[131,183], however such complications, even if with a lower prevalence, can be present in well-controlled patients. PD has been historically considered the sixth diabetic complication of diabetes [184]. Analysis of 4343 participants, aged 45-90 years, of the National Health and Nutrition Examination Study III showed a 2.9–fold increased risk of PD in poorly controlled diabetes compared to individuals without diabetes [185]. Similarly the degree of periodontal health was found to be lower in patients with poorer glycemic control from a number of observational studies [186,187,188]. Pima Indians with poor glycemic control had an 11-fold increased risk of bone loss progression compared to controls with no diabetes or well-controlled diabetes[182]. However, the importance of glycemic control is not reported in all the studies available[189,190].

1.5. Inflammation

Inflammation is a human phenomenon firstly described by the Roman doctor Cornelius-Celsus in the 1st century AD as the sum of four clinical events: *Rubor* (redness due to hyperaemia), *tumor* (swelling, caused by increased permeability of the microvasculature and leakage of proteins into the interstitial space), *calor* (heat associated with the increased blood flow and metabolic activity of the cellular mediators of inflammation), and *dolor* (pain, in part due to changes in the peri-vasculature and associated nerve endings). Much later, in 19th century, Augustus Waller and Julius Cohnheim explained inflammation and its physiological basis[191]. They suggested that the acute inflammatory response consists of the migration of leukocytes from the blood vessels, vasodilation and leakage of plasma into the interstitial space. In the middle of the 19th century, a fifth postulate of inflammation termed *functio laesa* was introduced by Rudolph Virchow to identify organ dysfunction linked to the inflammatory process[192]. The introduction of the concept of cellular phagocytosis and of the theory of cellular immunity by Elie Metchnikoff in the late 19th century represented the next milestone in our understanding of the inflammatory process emphasizing the beneficial aspects of inflammation in host defense and tissue homeostasis[193]. Subsequently, different classes of serum components as regulators of the inflammatory response were identified. Over the recent decades, the introduction of high sensitivity assays for inflammatory markers has greatly increased our understanding of the pathophysiological mechanisms involved in inflammatory responses. Inflammation not only acts as first line of defense of the human body

against external agents but also represents an adaptive response to acute or chronic alteration to its internal homeostasis.

1.5.1. Acute and chronic inflammatory responses

Acute inflammation represents an adaptive response to abnormalities due to endogenous or exogenous alterations[194]. Its aim is to allow the host system to reach homeostasis. According to the level of the alterations, the inflammatory response may involve only tissue resident cells or, in cases of a higher level of stress/damage, a more structured intervention with the expression of a range of inflammatory mediators and the activation of various immune cells. The chronic inflammatory response is required when the abnormalities duration is prolonged. It causes changes in many physiological processes inevitably related to adverse side effects. Subsequently, the initial beneficial protective response can result in further alterations to the body functions. The chronic inflammatory response, for its less aggressive component but prolonged duration, is considered a major contributor to various medical conditions[195]. For instance, a chronic inflammatory state is believed to contribute to the alteration of the hormone insulin production leading to type 2 diabetes and to the onset and progression of atherosclerosis[196].

1.5.2. Inflammatory pathways

The mechanisms involved in any inflammatory pathway comprise four main components: inducers, sensors, mediators, and effectors[197]. An inducer is any trigger that stimulates a sensor to product inflammatory mediators. The effectors are any tissue or organs involved in the inflammatory response.

1.5.2.1. Inducers and sensors

They can be divided into two broad categories depending on their microbial or non-microbial source. In addition, the non-microbial inducers are subdivided in two classes: Allergens, irritants, foreign bodies and toxic compounds as one group and a second category represented by molecules deriving from tissue or organ damage. However the inducers involved in numerous inflammatory conditions such as obesity, atherosclerosis, neurodegenerative diseases, and cancer remain still unknown. Specific cells of the innate or adaptive immunity recognize the presence of the inducers. Subsequently, cytokines, antibodies and other inflammatory molecules are released in order to resolve the alterations eliminating, if possible, the inducer.

1.5.2.1.1. Microbial inducers

Pathogen-associated molecular patterns (PAMPs), a variety of conserved molecular patterns commonly expressed on pathogenic or commensal species but not in mammals[198], allow the recognition of microbial inducers by different scavengers or toll-like receptors (TLR) of the immune system cells such as macrophages or mast cells[199]. Lipopolysaccharides (LPS), surface phosphatidylserine, aldehyde-derived proteins, or modified forms of classical risk factor for atherosclerosis, including low-density lipoproteins (LDL) modified by oxidation or glycation are examples of PAMPs. Interacting with either TLRs or scavengers receptors can cause the release of various inflammatory mediators in the first case or endocytosis and lysosomal degradation in the latter[200,201]. The production of mediators triggers an inflammatory exudate consisting of plasma proteins and white cells, mainly neutrophils, that reach the site of the invasion interacting with the adhesion proteins, selectins, expressed by the activated endothelium[202]. The neutrophils can be activated either by direct contact with the microbial inducers or with the mediators secreted by the tissue-resident cells.

In order to eliminate the inflammatory stimulus the neutrophils can produce a range of molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNO), proteinase 3, cathepsin G and elastase[203]. However, the substances released are not specific for the pathogens therefore can cause collateral damages hitting the host. The inflammatory molecules released can vary according to the type of invasion. For instance, viral infections stimulate the production of interferons and the activation of cytotoxic lymphocytes meanwhile parasites lead to histamine, interleukins release by basophils and mast cells[204,205].

1.5.2.1.2. Non-microbial inducers

Different groups represent this category. Allergens, irritants, toxic compounds and foreign bodies compose the first one[191]. For both allergens and irritants the inflammatory reaction consists of the activation of basophils and mast cells[206]. Foreign bodies involve mainly the macrophage phagocytosis[207]. The second group consists of intra- and extra-cellular molecules that in normal condition are contained in intact tissues and can be exposed after acute damages. For instance, the endothelium, after a vascular injury, regulates the plasma proteins and platelets migration to extravascular spaces[202]. The contact between collagen various components of the vascular extracellular matrix activates a plasma-derived inflammatory regulator, factor XII, triggers multiple proteolytic cascades generating inflammatory mediators such as the kallikrein-kinin, coagulation, fibrinolytic and complement cascades. In addition, the platelets contact with collagen stimulates the production of thromboxanes and serotonin[191].

1.5.2.2. Mediators

On the base of the biochemical properties expressed, there are seven categories of inflammatory mediators: Cytokines, chemokines, vasoactive amines and peptides, complement components, lipid mediators, antibodies and enzymes. Their impact on effectors may determine a variety of changes that will result in the clinical manifestations of the inflammatory response[208].

1.5.2.2.1. Cytokines

The term cytokine was firstly introduced on 1974 to define a group of proteins intervening in the inflammatory response[209]. They were initially believed to be released only by the traditional immune system cells and therefore called "lymphokines" or "monokines" according to the cell population source. The discovery that identical proteins could be produced by many other groups of non-immune cells brought to the definition by Balkwill and Burke in 1989 of cytokines as "one term for a group of protein cell regulators, variously called lymphokines, monokines, interleukins, interferons which are produced by a wide variety of cells in the body, play an important role in many physiological responses, are involved in the pathophysiology of a range of diseases, and have therapeutic potential"[210]. According to our current knowledge, cytokines comprise more than 50 molecules with molecular weights ranging from 8 to 40,000 dalton, divided in several classes (Table 7)

Table 7 Cytokines classes
- Interleukins.
- Tumor necrosis factors (TNF).
- Interferons (IFN).
- Colony stimulating factors (CSF).
- Transforming growth factors (TGF).
- Chemokines.

Depending on the structural homology of their receptors they can also be divided in Class I and II. Cytokines have also some specific features (Table 8)

Table 8 Cytokines features

- Pleiotropic action: triggering several different cellular responses depending on cell type, timing, and context.
- Synergic action: the association of two or more cytokines amplifies their activity or induces the expression of other cytokine receptors.
- Redundancy action: more cytokines may mediate similar functions making difficult to ascribe a particular effect to a single cytokine.
- Antagonism action: some cytokine can inhibit the effect of others.
- Cascade induction: a single cytokine can induce a target cell production of more cytokines that can stimulate additional cell cytokines production.
- Autocrine, paracrine or endocrine action.
- Share cytokine receptor subunits.

The main producers of cytokines are lymphocytes T_H and macrophages during the regulation of the immune response. The biological processes in which cytokines are involved are the innate and adaptive immunity, inflammation, hematopoiesis, cell growth and wound healing[209].

1.5.2.2.1.1. Pro-inflammatory cytokines

Some cytokines are involved in the promotion of the transient or perpetrated inflammatory response. The genes coding for their synthesis are up regulated during inflammation. Therefore they are classified as pro-inflammatory mediators[211].

1.5.2.2.1.1.1. Interleukin-1 (IL-1)

To date, eleven members represent the IL-1 family of which seven are categorized as pro-inflammatory mediators (IL-1α, IL-1β, IL-18, IL-33, IL-36a, IL-36b, IL-36c)[212]. Even detected at low concentration in the human body, they have a huge inflammatory effect. IL-1α and –β are two agonist forms with similar effects and IL-1 receptor antagonist (IL-1ra) competitively inhibits the binding to their receptors. The balance between IL-1β and its receptor antagonist (IL-1ra) level influences its relative physiologic or pathophysiologic effects[213]. The surface of their target cells has two receptors (IL-1R1 and IL-1R2). IL-1 seems to be central in the defense from infection. Some of them, such as IL-1β and IL-18, are released in a form of inert pro-peptide that require cleaving to bind their receptor and have a biological effect. Pro-IL-1β can be cleaved by inflammasome activation, neutrophil production of serine proteases (i.e. proteinase 3 and elastase) and mast cell-derived serine proteases (i.e. chymase). IL-1 is a major mediator in inflammatory, infectious and degenerative disease (Table 9). IL-1β is released by hematopoietic cells including blood monocytes, tissue macrophages, skin dendritic cells, and brain microglia, activated complement components, other cytokines (such as TNF-a), and IL-1 itself. IL-1 can be produced following metabolic alterations and during atheroma formation[214].

Table 9 IL-1 Effects
- Pyrogenic.
- The production of acute phase reactants in the liver.
- Increase vascular permeability and endothelium adhesion molecules.
- T/B-cell activation.
- Fibroblast proliferation.
- Platelet production.
- IL-6 and chemokines induction.

1.5.2.2.1.1.1.1. IL-1 and periodontitis

Robust evidence link IL-1 to the development of periodontitis[215]. It is detected in higher level in periodontal lesions. LPS or other bacterial components promote its production. A genetic polymorphism of IL-1 is associated with higher susceptibility to periodontitis[216]. Briefly IL-1 has a central role in the homoeostasis of periodontal tissues during a chronic inflammatory. IL-1 is associated with amplification of the inflammation response and linked directly to soft and hard tissue destruction.

1.5.2.2.1.1.1.2. IL-1 and atherosclerosis

A potential role of IL-1β in the atherogenesis has been investigated in both preclinical studies and animal models. The vascular endothelium release pro-coagulant mediators in presence of IL-1β and in experimental models of atherosclerosis, a slower progression of atheroma formation has been reported when IL-1 is not produced. In

addition, cholesterol can promote IL-1 release activating the NLRP3 inflammasome. In humans, circulating levels of IL-1 β are associated with the presence of traditional risk factors including hyperlipidemia, diabetes mellitus, smoking and hypertension. A polymorphism in IL-1ra known to increase levels of IL-1ra was associated with decreased mean coronary artery plaque area. In addition, the inhibition of IL-1β in subjects with high cardiovascular risk has significantly reduced inflammation supporting the therapeutic potential of its inhibitors[217].

1.5.2.2.1.1.1.3. IL-1 and diabetes

Evidence from human and animal studies suggest that the pathogenesis of T2DM could be related to an imbalance between IL-1 and its antagonists. Deregulation of beta-cell production of IL-1β could be due to glucose-induced activation of the caspase-1 inflammasome and by a decrease in the synthesis of beta-cell IL-1Ra. IL-1 antagonists seem to have beneficial effects on glycaemia and and b-cell function in T2DM[218].

1.5.2.2.1.1.2. Interleukin-6 (IL-6)

IL-6 is a cytokine released by various cell such as macrophages, neutrophils, keratinocytes, fibroblasts and endothelial cells in response to infectious or traumatic stimuli. IL-6 can stimulate different biological processes (Table 10)

Table 10 IL-6 Effects
- Pyrogenic.
- The production of acute phase reactants in the liver.
- Increase vascular permeability.
- T/B-cell activation.
- Immunoglobulin synthesis.
- Platelet production.

IL-6 is involved in both the initiation and resolution of the inflammation; for instance, it induces the acute-phase reactants release but also down regulates the neutrophil recruitment and the expression of pro-inflammatory cytokines.

1.5.2.2.1.1.2.1. IL-6 and PD

IL-6 has been detected in endothelial cells, fibroblasts, and macrophages of subjects affected by periodontitis. Its production by fibroblast of the human periodontal ligament is stimulated by specific oral pathogens. Periodontitis seems to be associated with increased level of IL-6 in saliva, GCF and serum. In addition, periodontal therapy has a double effect on IL-6 production increasing its presence transiently and reducing it on the long term. Therefore, to date, the main role of IL-6 in periodontitis pathogenesis is the modulation of the response to the bacterial challenge that leads to the local and systemic inflammatory response. An excessive IL-6 release has detrimental effect on the periodontal tissues, favoring connective tissue degradation and bone resorption.

1.5.2.2.1.1.2.2. IL-6 and atherosclerosis

Considering the multiple biological effect of IL-6, its actions can intervene in different stages of the atherosclerotic inflammatory process. In the development of the atheroma, endothelial cells respond to IL-6 production releasing chemokines and increasing surface adhesion molecules. In addition, the differentiation of T-cell in T-helper contributes to the inflammatory process. Inducing the expression of the monocyte tissue factor IL-6 has also pro-coagulant activity. Infections could be the main trigger for the IL-6 production, specifically respiratory tract infections, are associated with a transient increased risk for myocardial infarction, within 3 days of their onset. Chronic inflammatory conditions, such as rheumatoid arthritis, are related

to an increased cardiovascular risk. The main reason for the increased risk of mortality in rheumatoid arthritis is attributed largely to cardiovascular death. The injection of IL-6 to mice with fatty lesions determines their 5.1 fold increase. IL-6 release results from acute infection, chronic inflammation, obesity, and physiological stress causing inflammatory response, acute-phase reactants, and increased coagulation. It seems that having a variant in the IL-6 receptor leads to a decreased risk of CVD, therefore targeting IL-6 for the prevention of CVD could be a strategy to investigate in further studies.

1.5.2.2.1.1.2.3. IL-6 and diabetes

A higher concentration of IL-6 had been detected in T1DM[219] compared to controls. In addition, some evidence suggest that IL-6 participates in the initiation and acceleration of the chronic inflammation process and could contribute to the development of diabetic micro- and macro-vascular complications[220].

1.5.2.2.1.1.3. Interleukin-8 (IL-8)

IL-8 is a chemokine belonging to the CXC subfamily. It is secreted in the extracellular space in response to a variety of stimuli by a wide range of nucleated cell types but being monocytes and macrophages its main source. It is primarily involved in the recruitment of macrophages and neutrophils by means of a chemotactic gradient that attracts the inflammatory cells towards the area of increased chemokine concentration. Additionally, IL-8 has the function of activating neutrophils and monocytes. It can resist to temperature, proteolysis, acidic environment and it is relatively long-lived in sites of acute inflammation remaining active up to weeks after the onset of the inflammatory process. This is in contrast to the majority of the

inflammatory cytokines, which are not normally detectable in few hours after their release. IL-8 is also highly sensitive to anti-oxidants that reduce its gene expression.

1.5.2.2.1.1.3.1. IL-8 and PD

IL-8 has been associated with the pathogenesis of periodontitis and specifically with the recruitment and activation of neutrophils. Bacterial byproducts have demonstrated to affect both the production and the activity level of IL-8. It can be released in periodontal lesions by activated polymorphonuclear leukocytes, epithelial cells and gingival fibroblasts.

1.5.2.2.1.1.3.2. IL-8 and atherosclerosis

IL-8 has been detected in sites of vascular injury suggesting its potential contribution to the pathogenesis of the atheroma. In vitro and animal models reported that IL-8 was highly represented in the human arterial atherosclerotic wall compared with normal intima. In addition, macrophages were identified as the main source of IL-8 in atheroma. An observational study suggested that increased levels of IL-8 are associated with a higher CVD risk in low-risk individuals, independently of the traditional risk factor.

1.5.2.2.1.1.3.3. IL-8 and diabetes

Serum IL-8 profile in T2DM is significantly higher compared to the healthy population and varies according to the level of metabolic control. Therefore IL-8 could be involved in the development of late diabetic complications[221].

1.5.2.2.1.1.4. Interleukin-12 (IL-12)

IL-12 family includes Il-12, -23, -27 and -35. IL-12 is a mainly pro-inflammatory cytokine released by antigen presenting cells (APC) in response to microbial components. Its functions include the induction of T-bet and control of the differentiation of naive T cells into IFN-g-producing Th1 cells. In addition, it induces the release of IFN-γ by natural killer cells and innate lymphoid cells (ILCs).

1.5.2.2.1.1.4.1. IL-12 and PD

IL-12 has a major role in the initiation and enhancement of gingival inflammation. The importance of IL-12 is not limited to the onset but also may contribute to the maintenance of the immune response since Th1 responses rapidly decrease in the absence of IL-12. Increased levels of IL-12 were detected in both serum and GCF of subjects affected by periodontitis. The cysteine proteinases (gingipains) released by P. Gingivalis can hydrolyze IL-12 and lower the IFN-γ production. Inactivating IL-12 may also affect the cytokine balance in periodontal lesions leading to increased T-h activities.

1.5.2.2.1.1.4.2. IL-12 and atherosclerosis

IL-12 influences development of T lymphocytes to the Th1 and Th2 phenotypes and expressed in macrophages of human atherosclerotic lesions. Vaccination against IL-12 seems to reduce lesion formation in the carotid artery of mice and also daily administration of IL-12 enhances lesion formation in ApoE-/- mice. These evidences suggest a detrimental role of IL-12 in the atheroma development. In healthy individuals, IL-12 serum levels are associated with pulse wave velocity, a measurement for arterial stiffness and predictor for increased cardiovascular risk. In addition, its level is higher in subjects with angina pectoris or had myocardial infarction (MI).

1.5.2.2.1.1.4.3. IL-12 and diabetes

IL-12 has been related to the induction of Th1 cell-mediated autoimmune disease in multiple experimental models[222]. IL-12 administration seems to increase the level of pancreatic cells destruction and targeting its action has a protective effect in the development of T1DM in animal models. In addition, a new locus associated with human T1DM has been identified near the gene encoding IL-12 suggesting its role in the prediction of high-risk individuals for autoimmune diseases[223].

1.5.2.2.1.1.5. The tumor necrosis factor (TNF)

The tumor necrosis factor (TNF) superfamily is composed of 19 ligands and 29 receptors and contributes to a wide range of body functions. TNF-α is a cytokine initially identified for its ability to kill tumor cells in vitro. T cells can release TNF-α even if mononuclear phagocytes are its main cellular source. It is considered a pleiotropic pro-inflammatory cytokine in part through activation of the transcription factor NF-kB and has multiple biological effects (Table 11). Although TNF-α has physiologic roles including proliferation and differentiation of B cells, it also has been associated with pathologic conditions such as cancer, cardiovascular, neurologic, pulmonary, autoimmune, and metabolic disorders.

Table 11 TNF Effects
- Pyrogenic.
- The production of acute phase reactants in the liver.
- Increase vascular permeability and endothelium adhesion molecules.
- T/B-cell activation.
- Fibroblast proliferation.
- IL-6 and chemokines induction.

1.5.2.2.1.1.5.1. TNF-α and PD

Among its effects, TNF-α can promote collagenase production and bone resorption inducing periodontal tissues breakdown. It can activate osteoclasts and trigger the IL-1 production by macrophages. In addition, it acts synergistically with IL-1 causing bone loss. Microbial byproducts such as LPS can induce the release of TNF-α by peripheral blood mononuclear cells.

1.5.2.2.1.1.5.2. TNF-α and atherosclerosis

TNF-α has multiple actions on endothelial cells promoting coagulation and inflammation. It has been associated with an elevated risk of recurrent MI and cardiovascular death after a first MI. Its serum profile is related to ankle-brachial index and used to predict the severity of peripheral arterial disease. In addition, it correlates with the burden of atherosclerosis as assessed by carotid ultrasound among healthy middle-aged men.

1.5.2.2.1.1.5.3. TNF-α and diabetes

Several studies have reported elevated levels of TNF-α in individuals with insulin resistance and clinically diagnosed diabetes[224]. TNF-α seems to inhibit insulin transduction, and has an effect on glucose metabolism[225]. Perturbations of TNF-α metabolism may affect the onset of type 2 diabetes mellitus and the progression of the disease.

1.5.2.2.1.1.6. Interferons (IFN)

Interferons are key cytokines involved in the anti-viral response, firstly described in 1957 as an antiviral agent during studies on virus interference. To date, three types of INFs have been discovered (types I, II, and III). Type I IFN is represented by 14 IFN-α

species and a single species of IFN-β, -ω, -ε, -κ. IFN-γ is the only representative of the type II. It is released primarily by T-cells in the adaptive immune response. In addition, natural killers (NK) cells and APCs such as monocytes, macrophages and dendritic cells can secrete IFN-γ during the early host defense against infection under IL-12 and IL-18 regulation. Type I and type II IFN often have a synergic role in the activation of innate and adaptive immune responses involved in the antitumor immunity and the elimination of viral infections.

1.5.2.2.1.1.6.1. IFN-γ and PD

A significant increase in the level of IFN-γ has been detected in periodontal lesions compared to healthy sites in patients with chronic periodontitis[226]. Therefore, IFN-γ could contribute be involved in the stimulation of the phagocytic activity of the host cells and amplification the innate immune response to the periodontal pathogens[227,228].

1.5.2.2.1.1.6.2. IFN-γ and atherosclerosis

IFN-γ has various effects on endothelial cells. It can increase ICAM-1 expression and induce the synthesis of proteins of alternative complement pathway. It seems to inhibit endothelial cells growth and increase adhesion molecules specifically for lymphocytes. IFN-γ also modulates the expression of TNF-α. Macrophages are abundant in the atherosclerotic lesion and are activated on IFN stimulation. IFN-γ stimulates macrophages toward a pro-inflammatory phenotype. Activated macrophages can also secrete IFN, resulting in leukocyte attraction to the atheroma. In addition blocking IFN and its signaling leads to a decreased macrophage accumulation. VSMCs proliferation has a beneficial effect on the atheroma stabilization. IFN-γ seems

to inhibit their proliferation and collagen I and III production increasing the plaque vulnerability.

1.5.2.2.1.1.6.3. IFN-γ and diabetes

Evidence reports the role of IFN-γ in the increased expression of pancreatic derived factors suggesting its participation in the pathogenesis of diabetes and pancreatic cells death[229].

1.5.2.2.1.2. Anti-inflammatory cytokines

Among the cytokine network, potentially with the exception of IL-1, all the anti-inflammatory molecules might have a pro-inflammatory role. The effect on the inflammatory response will depend on the timing of the release, the balance with antagonists, the receptor density and the tissue responsiveness (Table 12).

Table 12 Major anti-inflammatory cytokines

Cytokine	Cell source	Main activities
IL-1ra	Monocyte/macrophage dendritic cells	Specific inhibitor of IL-1α and IL-1β mediated cellular activation at the IL-1 cellular receptor level
IL-4	T cells (Th2), mast cells, B cells, stromal cells	Promotes Th2 lymphocyte development; inhibition of LPSinduced proinflammatory cytokine synthesis
IL-6	T cells, B cells, monocytes, PMNs	Inhibition of TNF and IL-1 production by macrophages
IL-10	Monocyte/macrophage, T cells (Th2), B cells	Inhibition of monocyte/macrophage and neutrophil cytokine Production. Inhibition of Th1-type lymphocyte responses
IL-11	Stromal cells, fibroblasts	Inhibits proinflammatory cytokine response by monocyte/macrophages and promotes Th2 lymphocyte response
IL-13	T cells (Th2)	Shares homology with IL-4 and shares IL-4 receptor; attenuation of monocyte/macrophage function
TGF-β	Expressed in many cell lines	Inhibition of monocyte/macrophage MHC class II expression and proinflammatory cytokine synthesis

1.5.2.2.1.2.1. Interleukin-10 (IL-10)

IL-10 represents the main anti-inflammatory cytokine of the human immune system being a strong inhibitor on the Th-1 inflammatory mediators such as IL-1 and IFN-γ and monocytes/macrophages cytokines including TNF-α, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, MIP-1α, and MIP-2α. In addition, IL-10 reduces the surface expression of TNF receptors and induces the shedding of TNF receptors into the systemic circulation. IL-10 is mainly released by Th2, monocytes and B cells. After an experimental administration of IL-10 to human volunteers subsequent to endotoxin injection, individuals presented with less inflammatory symptoms when compared to placebo control.

1.5.2.2.1.2.1.1. IL-10 and PD

The anti-inflammatory action of IL-10 can reduce the synthesis of IL-1, IL-6, TNF-α, nitric oxide, gelatinase and collagenase. Therefore it regulates the bone homeostasis in both physiologic and pathologic conditions such as periodontitis. The lockout of IL-10 may result in accelerating alveolar bone absorption and decreasing bone formation and in IL-10 knockout mice there is a clear role in the development of periodontal disease. A polymorphism of the IL-10 gene could be at the base of a higher susceptibility to PD. The haplotype ATA of IL-10, causing low production, is considered a risk indicator for generalized aggressive periodontitis. In addition, the detrimental effect of smoking on the periodontum could be induced also by a down-regulation of IL-10 release.

1.5.2.2.1.2.1.2. IL-10 and atherosclerosis

IL-10 seems to be a potent anti-atherogenic molecule, therefore its serum levels have been explored in many trials investigating its potential cardiovascular risk prediction.

Elevated IL-10 serum profile was related with a significantly improved outcome independently of elevated troponin levels suggesting low serum IL-10 level as a marker of plaque instability. In addition, increased IL-10 serum levels are associated with improved endothelial function in patients with elevated CRP serum levels. Therefore the pro- and anti-inflammatory balance could be a key factor in the endothelial homeostasis.

1.5.2.2.1.2.1.3. IL-10 and diabetes

Numerous evidence report a significant difference between IL-10 serum levels in T2DM compared to healthy controls. Therefore, it has been suggested that low levels of IL-10 could be considered as a risk factor for T2DM[230].

1.5.2.2.2. Soluble adhesion molecules

Adhesion molecules are a group of cell surface glycoproteins expressed on a wide range of cells. They mediate the attachment of cells to each other, regulating the recruitment of immune cells to the inflammatory site. Their soluble form detectable in the bloodstream derives from the cleavage of the extracellular portion expressed on the activated cell membrane. However some types, such as P-selectin are also produced in a soluble form.

1.5.2.2.2.1. Intercellular adhesion molecules-1, 2, 3 (ICAM-1, ICAM-2, ICAM-3)

ICAM-1 (CD54) and ICAM-2 (CD102) are membrane glycoproteins belonging to the Immunoglobulin family. They are both expressed by endothelial cells, neutrophils, platelets and different leukocytes sub-population. ICAM-1 is also present on fibroblasts, epithelial cells and pericytes and located only on the endothelial surface. ICAM-2 is present also on the cellular junctions. The endothelium can increase the expression of ICAM-1 following inflammatory stimuli such as TNF-α and IFN-γ or

bacterial byproduct as LPS. ICAM1 and ICAM-2 can both engage the neutrophil integrins modulating their transmigration. ICAM-3 is expressed on different leukocytes populations but not on normal or activated endothelium therefore it intervenes in the early leukocytes-leukocytes interactions such as B cell activation mediated by T cells or T cells activation by APCs.

1.5.2.2.2.2. sE and sP-selectins

E-selectin is highly selective and is only transiently expressed by the activated endothelium facilitating the rolling of leukocytes along the endothelial layer as a prelude to leukocyte adhesion. Its soluble form has been detected in the supernatants of TNFα and IL-1 stimulated endothelial cells and it is studied as diagnostic and prognostic markers in a variety of infectious diseases since it is released following endothelial damage both in vitro and in vivo. In chronic inflammatory conditions it is up-regulated and declines following the remission inducing treatment.

P-selectin is a preformed molecule which is stored in the granules of platelets and the Weibel-Palade bodies of the endothelium, and is involved in the interaction between platelets, the endothelium and leukocytes, thus playing a key role in vascular inflammation. The soluble form has been shown to be up-regulated in active inflammatory conditions.

1.5.2.2.3. Markers of vascular damage

1.5.2.2.3.1. sThrombomodulin (sTM)

TM is a glycoprotein presents on the majority of the endothelial cells representing approximately 60% of the thrombin-binding site and responsible for the anticoagulant features of the endothelium. It is also found in blood plasma, platelets, neutrophils, monocytes, urine, and placenta. TM regulates the blood coagulation contributing to

the maintenance of its liquid state avoiding intravascular coagulation. Once bound to TM, thrombin does not favors the fibrinogen conversion into fibrin and platelet aggregation. In soluble form, sTM is detectable in plasma and urine and it is related mainly to endothelial damage and not to its physiological activation. Therefore sTM is a validated marker of endothelial damage directly correlating with its degree. Its profile has been reported to be elevated in multiple pathological conditions including diabetic microangiopathy, systemic lupus erythematosus (LES), peripheral and coronary atherosclerosis and cancer.

1.5.2.2.4. Acute phase reactants (APRs)

APRs are a class of plasma proteins detectable in different inflammatory conditions including infections, autoimmune disorders, cancer and trauma. Pro-inflammatory cytokines such as IL-1, IL-6, TNF-α stimulate the APRs hepatic production that represents their main source however macrophages, endothelial cells, fibroblasts, and adipocytes can participate to their synthesis. This category includes various molecules such as C-reactive protein (CRP) serum amyloid A (SAA), ceruloplasmin, fibrinogen, alpha-1 antitrypsin, complement proteins and ferritin. The response varies among the APRs having CRP and SAA increasing up to few thousands folds and fibrinogen only few folds. CRP is probably the most common APR evaluated in clinical practice.

1.5.2.2.4.1. C-reactive protein (CRP)

CRP is a plasma protein produced mainly by the liver hepatocytes during the acute phase reaction. Its properties range from the stimulation of cytokine production, complement activation, recruitment and activation of inflammatory cells and promotion of atherogenesis. Its plasma profile in healthy individuals is normally between 1 to 3mg/L but it can reach 10mg/L. It recognizes and binds microbial surface

polysaccharides activating the complement pathway and opsonizes them to induce phagocytosis.

1.5.2.2.4.1.1. CRP and PD

Numerous studies suggest that PD is associated with an increased CRP level. Its biological plausibility is related to the higher local production of inflammatory cytokines in PD that in turn triggers a systemic inflammatory response. A recent systematic review of 23 clinical trials reported that CRP reduction was obtained following periodontitis treatment when other co-morbidities were identified (diabetes and cardiovascular disease) but not inclusion of smoking and obesity.

1.5.2.2.4.1.2. CRP and atherosclerosis

CRP has been identified as a risk factor for future cardiovascular events in the asymptomatic population. It has been observed in atheromas and damaged vessels that contribute in a small extent to its release. A large trial on healthy individuals with normal cholesterol level but elevated CRP comparing the administration of statins to placebo has reported that lowering level of CRP is associated with a reduced risk of future cardiovascular events.

1.5.3. Inflammatory resolution and chronic inflammation

The inflammatory cascade, consequent to cellular and tissue damage, aims to tissue repair and to counteract pathogenic invasions. Its duration should be limited to its purpose however, the removal of the pro-inflammatory mediators is only a part of the resolution process since it aims to restore the normal function and architecture of the damaged tissue. It is now evident that the pathways involved in the termination of the inflammatory response are numerous and complex[231]. Following the resolution of the mediators release and the leukocytes infiltration, the tissue repair and homeostasis

are the main aims. Vasculature, extracellular matrix and various cell types are involved in this process. A dysfunctional vasculature remodeling may result in atrophy or tissue fibrosis interfering with the organ function representing a major problem in inflammatory processes of the arteries. Therefore, a persistent inflammatory condition and a deficient inflammatory resolution process cause chronic inflammation. This is a major contributor to the progression of multiple clinical conditions such as atherosclerosis[107], arthritis[232], neurodegenerative disease[233] and periodontitis[234]. The inflammatory inducers involved in the acute response can also trigger chronic reactions. Their source can be microbial or not and the pathways involved depend on the inflammatory trigger. T cells normally replace neutrophils and macrophages infiltrate if microbial inducers are not completely removed[235]. The switch from the innate to the adaptive response involves the interaction with the surface antigens of the inducers[236]. The initiation of the adaptive immunity starts when immature dendritic cells ingest and degrade a pathogen[237]. The activation of the dendritic cell transforms it in an effective APC[238]. Their role is to carry the microbial antigens to peripheral lymphoid station interacting with T lymphocytes. The cytokines secreted by the APC have an action on both innate and immune activities. T cells react to the antigen recognition activating cytotoxic T cells and stimulating the antibodies production by B cells[239]. T cells have also the feature of differentiating in either T helper 1 cells (Th1) or T-helper2 (Th2) sub-phenotypes both involved in multiple inflammatory processes[240,241]. Th1 are involved in both the response to pathogens and hypersensitivity skin reactions. They secrete IL-2, TNF-α, and IFN-γ stimulating chemokines production, a link between innate and adaptive immunity[242]. If there is a continuous inflammatory induction not eliminable by macrophages and the adaptive immunity, the formation of granulomas or lymphoid tissues might follow[243]. A

granuloma is consequent to either persistent microbial exposure or non-microbial inducers. The macrophages encapsulate the inflammatory trigger with multiple layers counteracting the induction of further immune response. This condition might endure for several years, however the granuloma can reactivate with a consequent new inflammatory reaction such as in tubercular granuloma or endodontic lesions[244].

1.5.4. Free radicals and oxidative stress

Free radicals have the capacity to oxidize, extracting electrons, molecules involved in the body homeostasis[245]. They can be categorized in reactive oxygen species (ROS), the most important, nitrogen and chlorine species. Reactive species (RS) is used as a term to define true radical and reactive compounds that are able to from radicals. Antioxidants are molecules able to maintain a body balance between oxidative and reduction activities. Oxidative stress is a state in which the oxidant processes are not balanced by their counterpart either by higher production of radicals or reduction in antioxidants activity[246]. It is associated to potential cellular damage. Radicals can derive from exogenous and endogenous sources:

- Exogenous: ultrasound, ultraviolet light, ozone, smoking, exhaust fumes, radiation, infection, excessive exercise and therapeutic drugs

- Endogenous: superoxide formed from electrons leaking from the mitochondrial respiratory chain and radicals generated by host cells (phagocytes, osteoclasts and fibroblasts)

RS are produced by phagocytic cells following injury and inflammation of tissues as a mechanism to kill invading microorganism[247]. When inflammation becomes systemic as in inflammatory response syndrome there is loss of control of RS production leading

to non-discriminant injury of tissues and organs in the host[247]. Oxidative stress has been shown to cause secondary damage through delayed cellular death and inflammation[248]. Several diseases such as diabetes[249], hypertension[250], heart failure[251], Parkinson disease[252], renal disease[253], epilepsy[254], Alzheimer's[255] and other neurodegenerative diseases[256] have been linked to oxidative stress . Therefore, minimizing oxidative stress may prevent cellular death, decrease inflammation, and prevent some morbidity and mortality[257].

1.5.4.1. Mitochondrial respiratory chain and RS production

Within the cell, mitochondria represent the main source and, due to proximity to their production, first target of ROS. Mitochondria consume the majority of the cellular oxygen during the normal cell energy production. A small percentage of oxygen is converted to RS and, in certain individuals, defective mitochondria could produce an increased level of RS. The in situ generation of oxidant has promoted the production of antioxidants within the mitochondria such as mitochondrial manganese superoxide dismutase, copper ⁄ zinc superoxide dismutase, glutathione peroxidase and catalase. The protective activity of these enzymes may not be sufficient to neutralize all the ROS and specific biomolecules including nucleic acids, proteins and lipids could be exposed to oxidative damage.

1.5.4.2. Indirect mechanisms of ROS-induced inflammation

The indirect mechanisms by which ROS can cause an increased inflammatory burden lines on their ability to modify the function of the three main classes of cellular macromolecules: lipids, nucleic acids, and proteins (Figure 9).

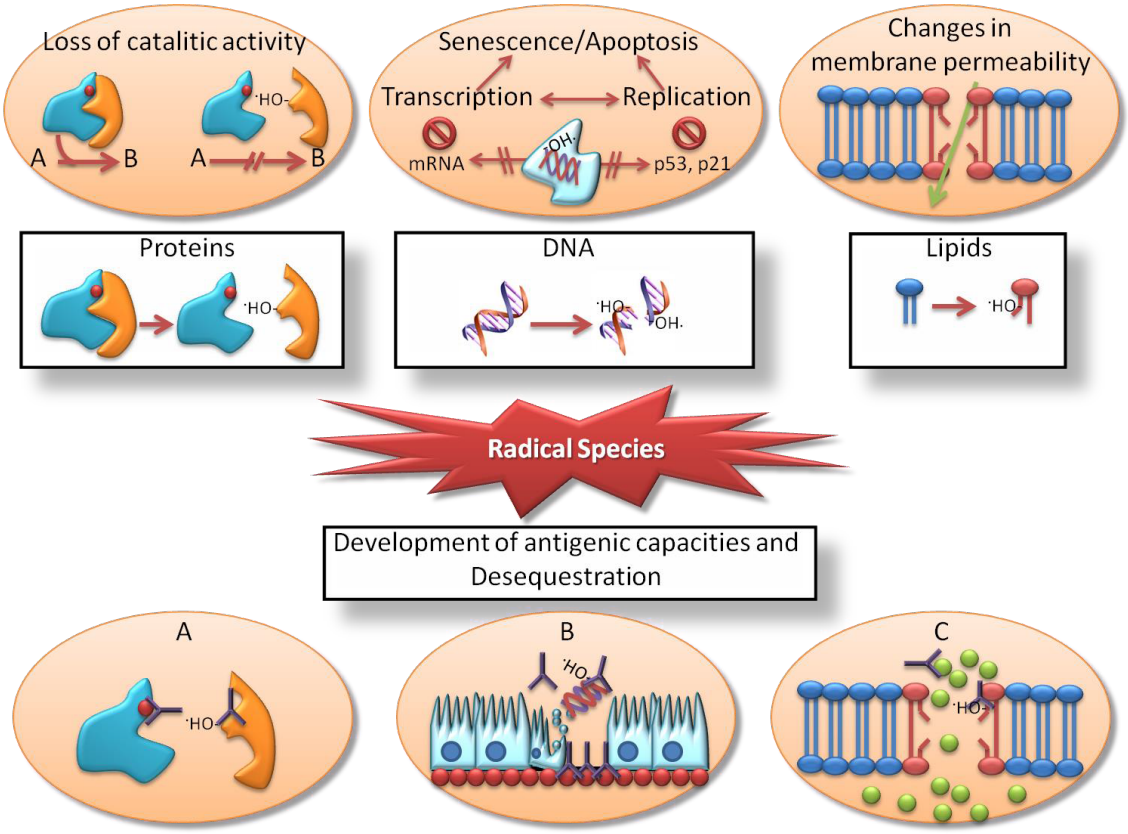


Figure 9 Oxidative mediated cellular damage

Each intracellular macromolecule can be damaged by increased bioavailability of oxygen radical species. Oxidative damage to: A) proteins leads loss of catalytic activity, B) nucleic acids leads to activation of the DNA damage response or modification of mRNA, causing arrest of cell cycle and cellular senescence or apoptosis, C) lipids leads to increase membrane permeability and loss of the trans-membrane homeostasis. These processes can lead to activation of the immune system, either because structural changes make macromolecules recognized as foreign bodies by the immune systems or because of decompartmentalization and exposure of antigens that normally are sequestered inside cells.

This can lead to tissue malfunction and associated up regulation of the inflammatory response by several mechanisms:

1. ROS can react with cell membrane fatty acids and form lipid peroxides, resulting in permanently impaired fluidity and elasticity of the membrane, and

consequent cell rupture[258] (Figure 9). Similarly, overproduced radicals can react with protein amino acids leading to their oxidation and cross-linking. Radical-protein reactions can permanently impair the function of important cellular and extracellular proteins, including enzymes and connective tissue proteins (Figure 9). It has been estimated that oxidized protein in old rats may comprise 30-50% of the total cellular protein. DNA is another macromolecule, which is highly susceptible to free radical attack. An oxygen radical interaction with DNA can break its strands or delete a base. This DNA damage can be a lethal event for an organism, as it can cause an irreversible arrest of cell replication (Figure 9). It has been estimated that, on average, more than 10,000 oxidative hits occur each day in the DNA of a single human cell[246].

2. Structural changes induced by free radical damage have the potential to transform inert macromolecules into potent inflammatory inducers. One example is the conversion of inactive low density lipoproteins (LDL) into their highly pro-inflammatory counterparts (ox-LDL), a process determined by oxidation of the lipid and protein components of the lipoproteins[107].

3. Functional changes induced by free radicals in membranes, proteins and nucleic acids lead to progressive cellular and tissue damage, followed by desequestration of endogenous antigens (Figure 9). For instance, increased exposure to oxidative stress seriously damages endothelial cells which not only become dysfunctional, but also lose integrity, progress to senescence, and detach into the circulation[202]. As previously discussed, sites of “disendothelization” represent potent activators of the Hageman factor, which interacts with components of the extracellular matrix and initiates the inflammatory response by stimulating the kallikrein-kinin, coagulation, fibrinolytic and complement cascades. Oxidative damage to intracellular

components has been suggested not only as the primary source of inflammation during aging but also as the most important pathway accounting for the aging process

1.5.4.3. Direct mechanisms of RS-induced inflammation

Over the last few years, it has become clear that an increased bioavailability of RS can induce chronic inflammatory responses by direct mechanisms. Several studies demonstrated that, the pro-inflammatory effects elicited by the oxidative damage to cellular macromolecules, gene expression of pro-inflammatory IL-1β, IL-6, TNF-α, cyclo-oxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS) are enhanced during aging by the redox-sensitive transcription factor nuclear factor-κB (NF-κB)[259,260]. Similarly, current research suggests a central role for RS of mitochondrial origin (mtRS) in mediating the increased inflammatory levels observed in the elderly[261]. While mitochondria are crucial for normal cell functions, their respiratory activity is coupled with release of high levels of RS which, in turn, act as signaling molecules, directly triggering pro-inflammatory cytokine production[262,263]. In order to prevent excessive accumulation of mtRS, cells developed a complex mechanism, known as mitophagy (more generally known as "autophagy"), to control the number of mitochondria within a cell by removing those which become dysfunctional and produce high amount of mtRS[264]. Autophagy is an essential cyto-protective pathway and consists in the formation of autophagosomes, double-membrane vesicles that sequester organelles, proteins, or portions of the cytoplasm, which then fuse with lysosomes[265]. As a result, the sequestered contents are degraded by lysosomal enzymes, and recycled as a source of energy[265]. Autophagy may occur either as a general phenomenon, for instance when cells lack nutrients and mobilize their energy reserves, or it can specifically target distinct cellular structures such as damaged mitochondria ("mitophagy")[266]. Inhibition of autophagy results in the

accumulation of damaged mitochondria in human cells, leading to an increase in the net amount of mtRS and pro-inflammatory cytokine production[263]. Interestingly, autophagy appears to decline with age, and gene expression of key regulators in the autophagic pathway (i.e ATG5 and ATG7) are reduced in aging individuals[267]. Additionally, major human age-related diseases characterized by an increased inflammatory burden such as Parkinson's and Alzheimer's disease have been linked to defects in mitochondrial autophagy[268]. This data strongly supports a possible contribution of mtRS to the age-dependent increase of the inflammatory burden. Interestingly, conditions that promote autophagy, such as caloric restriction and exercise, delay aging-associated degeneration[269], suggesting that autophagy exerts important roles also in controlling the evolution of aging. Stimulation of autophagy can increase the healthy lifespan in multiple model organisms including mice and primates[270], while experimental inactivation of genes required for the execution of autophagy is lethal at the whole-body level, whereas tissue-specific knockouts induce organ-specific degenerative changes[271].

1.5.5. Human models of acute systemic inflammation

Systemic inflammation is involved in the pathogenesis of several acute and chronic conditions[272-276]. In addition, sepsis, the acute systemic response to infections, is responsible for a large number of deaths in intensive care units[277]. In order to achieve a better understanding of the pathways involved in the inflammatory response, human in vivo models of systemic inflammation have been introduced to study the inflammation and its role in acute and chronic diseases[278].

1.5.5.1. Intravenous injection of lipopolysaccharide (LPS)

Bacterial components have been administered to humans for therapeutic purposes to treat several conditions such as inflammatory bowel diseases, autoimmune disorders, cancer, metabolic diseases and obesity or to combat bacterial and viral infections[279]. In addition, the bacterial byproducts can be used to study the human innate immune response in vivo. Recognition molecules present in the bloodstream or on the cell surfaces can initiate the inflammatory process by opsonic activity, endocytosis or activating inflammatory cells[280]. The human endotoxin model represents the most common model of systemic inflammation because of its high reproducibility of effects; purified LPS from Escherichia coli or other Gram-negative bacteria is administered intravenously to healthy volunteers allowing the observation of human inflammation in a dose-dependent manner qualitatively similar to the early phase of sepsis[281,282]. Within 1 hour after the administration, volunteers experience varying degrees of flu-like symptoms such as chills, headache, myalgias and arthralgias, nausea and photophobia with a large inter-individual variation and in a dose-dependent fashion[283]. These symptoms tend to attenuate within 2 to 6 hours in parallel with the reduction of the pro-inflammatory response. Tachycardia and a temperature increase of approximately 2°C are the most reproducible clinical findings. LPS triggers the production of pro-inflammatory mediators such as TNF-α and IL-1β, chemokines. Within the first hour after LPS administration, TNF-α, soluble TNF-α receptor, IFN-γ, and IL-6 appear in plasma. Of these, TNF-α shows a monophasic peak after 90 minutes, whereas IL-6 and IFN- γ peak after 120 minutes[284,285]. IL-1β peaks after TNF-α, but before IL-6[286]. The anti-inflammatory mediators are detectable subsequently response; IL-10 peaks 3 hours after the exposure to LPS[287]. Higher profile of chemokines such as IL-8, monocyte chemoattractant protein (MCP)-1, and neutrophil attractant protein

(NAP)-1 can also be detected subsequent to the administration of LPS[288]. The acute phase proteins profile rise on the following 12 to 24 hours[282]. Furthermore, the hypothalamic–pituitary–adrenal axis rise within 2–3 hours and the adrenocorticotrophic hormone, cortisol, and the adrenal androgen dihydroepiandrosterone profile increase acutely[289]. The endotoxemia model is not comparable to sepsis since it is not based on equivalent doses required for a similar response; in addition, the healthy subjects rather than high-risk populations and the LPS rather than living bacteria represent clear limitations of this model. However, it represents an opportunity to describe immunological, metabolic and physiological changes due to the inflammatory response.

1.5.5.2. Infusion of cytokines

Increased plasma levels of TNF-α and IL-6 have been related to several conditions such as sepsis, atherosclerosis and T2DM[290-293]. Therefore, intravenous injection or infusion of recombinant cytokines has been adopted to evaluate the body response. TNF-α infusion causes fever, pituitary and stress-hormone secretion together with an acute phase response. These effects and Infusion of IL-6 are comparable to the LPS model[294,295].

1.5.5.3. Typhoid vaccine

Salmonella typhi capsular polysaccharide vaccine injected into the gluteus muscle of healthy volunteers has been associated with higher total white cell count, progressive increase of IL-6 and IL-1Ra after the vaccination. Myalgia and headache were reported in few subjects and no fever or increased blood pressure were reported[296]. In addition the administration of 1.2g of aspirin to healthy volunteers before Salmonella typhi

capsular polysaccharide vaccine has a protective effect on the endothelial dysfunction following the mild systemic inflammation following the vaccine administration[297].

1.5.5.4. Strenuous exercise

The body response to physical exercise has some similarities with the inflammatory response detected after trauma or infections; therefore it has been proposed as a model of systemic inflammation and several studies have evaluated the inflammatory response following exercise-induced muscle damage[298]. The muscle damage following eccentric cycle ergometry triggers cytokines release[299]. IL-1β was elevated in the muscle tissue of individuals who performed 45 minutes of downhill running[300] and higher level of LPS was detected after a triathlon competition[301].

It is plausible that exercise could induce ischemia of the gastrointestinal system increasing gut permeability to allow penetration by bacterial toxins triggering the inflammatory response via immune cell response. Strenuous exercise seems to stimulate the secretion of cytokines in a similar manner to subclinical inflammation and sepsis[302] with the initial release of TNF-α and a subsequent rise of IL-6, IL-1β and IL-1ra. A 100-fold elevation of IL-6 mimic the increase of this cytokine during sepsis however, the 2.5 fold rise of IL-1β and TNFα and a lack of reproducibility reduce the applicability of this model.

1.5.5.5. Periodontal treatment

PD and its treatment could represent a model to study the inflammatory response in humans since periodontal infection has been linked with an increased level of systemic inflammation. PD can be described as a chronic low-grade infection mainly due to anaerobic gram-negative bacteria. Periodontal therapy is primarily based on the mechanical removal of the bacterial biofilm and hard deposits from the diseased

dentition. The consequent acute bacteremia and soft tissue damage could provide a model to study the transient host response. Following an intensive session of sub-gingival mechanical instrumentation under local anesthesia, the inflammatory response was assessed at day 1 and day 7 follow-up visits (Figure 10). An acute increase of inflammatory mediators such as TNF-α, IL-6 and CRP was observed. TNF-α profile was higher at day 1 visit. IL-6, CRP and Fibrinogen plasma levels were also increased 24 hours after periodontal treatment and returned to value similar to baseline only after 1 month. There was an increase in circulating neutrophils and monocytes. Lymphocytes decreased at day 1 and then increased 7 and 30 days after treatment. Furthermore, erythrocyte numbers, and hemoglobin concentration were lower 7 days after the intervention[303]. Periodontal treatment could then represent a therapeutic model to study the acute inflammatory response.

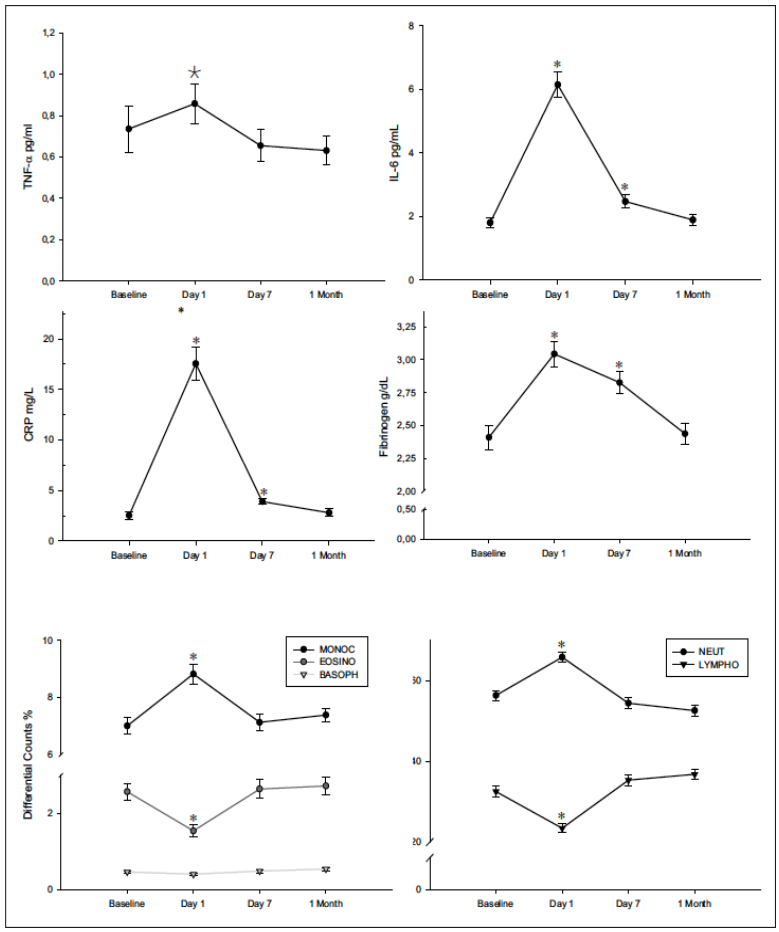


Figure 10 Host response following periodontal treatment.

1.6. Mechanisms of the association between Periodontal Disease, Cardiovascular Diseases and Diabetes

1.6.1. Periodontitis and Diabetes

The sub-gingival biofilm of periodontal patients does not seem to differ in presence of diabetes[190,304,305] suggesting that a dysfunction in the host inflammatory response may contribute to the increased prevalence and severity of PD observed in diabetes. Diabetes is related to an altered function of immune cells such as neutrophils, monocytes and macrophages[306]. Neutrophils features including adherence, chemotaxis, and phagocytosis can be impaired reducing their efficacy in eliminating bacteria in the periodontal lesions with a consequent increase in periodontal damage[307]. In addition, monocytes and macrophages exhibit an increased response to the bacterial challenge raising the release of pro-inflammatory mediators[308]. In diabetes, Porphyromonas gingivalis antigens trigger a higher quantity of TNF-α by blood monocytes compared to controls[308]. This evidence is supported by data from an animal model of diabetes in which P. gingivalis inoculation resulted in a prolonged inflammatory response due to TNF stimulation[309]. Furthermore, the GCF analysis of patients with PD and diabetes report an increased level of inflammatory mediators supporting this hypothesis[310]. It seems also that the GCF inflammatory profile relates to the level of glycemic control; GCF levels of IL-1β were approximately doubled if HbA1c levels were over 8%[311]. Alterations in the connective tissue metabolism could also be a contributor to the increased level of bone loss as hyperglycemia has been associated to impaired bone healing and turnover[312] and an increased rate of apoptosis in response to P. gingivalis infection[313]. Furthermore, high glucose level in

the GCF may affect the fibroblasts wound healing action required maintaining a healthy periodontium[314]. The micro-vascular complication of diabetes include abnormal growth and impaired healing of the vessels are detectable at a periodontal level[315]. The sustained hyperglycemia cause the proteins transformation in advanced glycation end products (AGEs) that can bind the collagen and accumulate on the intima of blood vessel contributing to the diabetic vascular complications[316,317]. Furthermore AGE can increase the basement membrane thickness of the microvasculature affecting its normal homeostatic transport properties. This change in homeostasis may alter wound healing responses to chronic microbial damage of the periodontium. Numerous evidence have suggested a potential impact of PD on the metabolic state in diabetes[318] worsening the glycemic control. Data from a 2-year longitudinal trial report that patients with diabetes and severe PD had a six-fold increased risk of worsening of glycemic control over time compared to controls[319] . Furthermore, an increased risk of other diabetic complications has been observed in a case-control study; 82% of diabetic patients with severe PD experienced at least one major cardiovascular, cerebrovascular, or peripheral vascular events compared to only 21% of controls[320]. A longitudinal trial examined the effect of PD on overall mortality and cardiovascular disease–related mortality in more than 600 subjects with T2DM reporting a death rate from ischemic heart disease was 2.3 times higher than in patients with no periodontitis or mild periodontitis. In addition, severe PD was associated with an 8.5 times higher mortality rate from diabetic nephropathy and a 3.5 times higher overall mortality rate from cardio-renal disease[321].

1.6.2. Periodontitis and Cardiovascular Diseases

The inflammatory mediators detected in both PD and CVD ha been proposed as a potential contributors to the link between these two conditions. The inflammatory

response triggered by the periodontal microorganisms could contribute to an increased risk of CVD onset, progression and severity through different pathways[9].

1.6.2.1. Increased systemic inflammatory mediators

Periodontal pati8ents have a higher systemic concentration of inflammatory mediators compared to controls.

1.6.2.1.1. Locally produced cytokines access to the circulation

One plausible explanation for this difference could be related to the access to the circulation of inflammatory cytokines and other mediators produced in the periodontal lesion[322]. Their higher level in the bloodstream could have an impact on other tissues distant from the periodontium such as the liver, stimulating an acute phase response. Consequently, the endothelium would up-regulate the adhesion molecules and promote the cytokine production contributing to the pathogenesis of the atheroma. However, there is no strong evidence, for the time being, supporting this hypothesis[323].

1.6.2.1.2. Bacteremia

A second potential mechanism could be related to the bacteremia associated with periodontitis; transient LPS concentration can be detected in the circulation. Furthermore, P. gingivalis in animal models stimulates inflammation in locations distant from the oral cavity [324-326]. Bacteria or their byproducts could be involved in the systemic response and in the exacerbation of the atherosclerotic lesions[323].

1.6.2.2. Antibodies

Additionally, specific cross-reactive antibodies induced by periodontal pathogens can increase the risk for atherosclerosis or accelerate its course by promoting the

endothelial inflammatory response, macrophages endocytosis of lipid and contrasting the anti-atherogenic actions of protective molecules. The immune response to microbial heat-shock proteins (HSPs) could represent a mechanism linking bacterial infections to atherosclerosis. In humans, HSPs transport protective proteins to the cell surface. During the inflammatory response, HSPs will be expressed by tissues and regulated by the immune system. Anti-HSP reactive antibodies can be detected in serum of patients with atherosclerosis and HSPs can interact directly with Toll-like receptors (TLRs) and thereby induce inflammatory responses in macrophages and endothelial cells. Bacteria can express antigens resembling human HSPs stimulating the production of antibodies and the T-cells activation. Therefore, molecular mimicry inducing cross-reactive cells and antibodies, may be a link between infection and atherosclerosis[327]. Periodontal pathogens such as P. gingivalis can express HSPs and stimulate macrophages to release inflammatory cytokines [328-330]. Fusobacterium nucleatum can also express HSPs, antibodies for its HSPs have been detected in periodontal patients[331]. Fusobacterium nucleatum HSPs could enhance foam cell formation, activate endothelial cells with increased monocyte adhesion and migration and promotion of coagulation[331]. Furthermore, animal models support a potential interaction of bacterial HSPs and promotion of atherosclerosis[332] supporting the role of a response to HSP, induced either by human or bacterial mediators, interaction with periodontal and atherosclerotic lesions.

1.6.2.3. Serum Lipids

Ultimately, PD is associated with elevated serum concentrations of potentially atherogenic lipids such as LDLs, triglycerides (TGs) and very low-density lipoproteins (vLDLs). In vitro studies suggest that PD is associated with changes in serum lipid

concentrations and lipid modification. The properties of serum lipids can be affected by infections; the response to bacteremia could interact with serum lipids and stimulate the liver cholesterol biosynthesis promoting dyslipidemia. Periodontal patients present higher cholesterol and LDL profile compared to controls[333,334]. Elevated LDL and lower HDL were observed in PD irrespectively of the smoking status[335]. In addition, increased serum levels of oxLDL and modified LDL were detected in PD[336,337].

1.7. Evidence that periodontal treatment improves CVD and Diabetes Outcomes

CVD namely coronary heart disease, stroke, congestive heart failure, and peripheral artery disease, became the leading cause of chronic disease morbidity and mortality in industrialized countries in the twentieth century[338]. CVD incidence is now increasing also in the developing countries as a consequence of the better control of infectious diseases and due to the obesity and diabetes epidemic[339]. A bulk of evidence is available on the beneficial effects of controlling a number of recognized CVD risk factor including hypercholesterolemia, hypertension, smoking[340] and sodium intake[341]. However the incidence of CVD is still increasing as controlling all recognized risk factors might not be sufficient at controlling CVD impact on the health of the general population. There is emerging evidence that inflammation plays a key role in the development of CVD from atheroma formation to its rupture and development of clinical events[293]. Several epidemiological studies have investigated and support an association between high levels of inflammatory markers an increased risk and progression of CVD[342]. A number of potential sources of inflammation have been investigated over the last 30 years. The possible etiological role of acute or chronic

infections on CVD has attracted great attention in recent years. In particular attention has been drawn on the potential impact of various infectious agents on systemic inflammation and autoimmunity and subsequently onset and progression of CVD[343]. PD shares most of its risk factors with CVD including age, gender, socio-economic status, diabetes, obesity, smoking and hypertension[344]. Evidence from prospective and cross-sectional studies supports a weak but consistent association between higher CVD risk and PD[161]. Individuals with PD on average presented with 14-15% greater risk of developing CVD from prospective trials whilst the odds increased to more than 100% when analysing case-control studies compared to healthy individuals[161]. A number of systematic reviews have confirmed these associations[158-160,164]. A recent cohort study report on CVD among 1400 dentate men aged 60 to 70 years, showed that severe loss of periodontal attachment conferred a statistically significant doubled risk of death compared with controls (15.7% versus 7.9%)[345]. The hazard ratio (HR) adjusted for age, smoking, diabetes, hypertension, body mass index, cholesterol, education and marital status and history of a vascular event 1.57 (95% CI 1.04–2.36). An increased mortality rate in individuals with a higher level of attachment loss had already been reported by other investigators[149,346]. According to these evidences the potential benefit of periodontal treatment in reducing CVD mortality represents the next logical step. A recent position paper of the American Heart Association reviewed the evidence available on the association between PD and CVD concluding that after 30 years of research, it is still not clear whether this link is causal[164]. The same paper called for new well-designed intervention studies investigating the impact of periodontal therapy on CVD outcomes. However a large number of small single-centre intervention trials have been published over the last 30 years focusing on various surrogate markers of

CVD. The aim of this review is to critically appraise the evidence available on the impact of periodontal treatment on CVD biomarkers and outcomes.

1.7.1. Treatment of Periodontitis and Diabetes outcomes

1.7.1.1. Effect of periodontal therapy on the glycemic control

The treatment of DM aims to primarily control the hyperglycemia since it is associated with a wide range of serious complications. Therefore, a regular monitoring of the glucose levels is required. The hemoglobin non-enzymatic reaction with glucose occurs continuously in erythrocytes. This bind is highly stable and last for approximately 123 ± 23 days, the whole life span of the erythrocytes[347]. Therefore, the assessment of HbA1c reflects the average of the glycaemia over the preceding 1–3 months [348]. Measurement of hemoglobin A1c (HbA1c) is recommended at least twice a year in stable patients and every 3 months in those who has changed therapy or are not meeting the treatment goals. The advised HbA1c target value for people with DM is <7.0%, with the healthy population below 6%. Recent data reported that only 36% of people with T2DM have HbA1c <7.0%[349]. Data from two large trials, the United Kingdom Prospective Diabetes Study (UKPDS) and the Diabetes Control and Complications trial, suggest that the risk of long-term complications can be lowered by an intensive treatment of hyperglycemia[131,183,350]. A 1% reduction in the HbA1c in the UKPDS was associated with a relative risk reduction of 21% for any diabetes-related endpoint, 21% for diabetes-related deaths, 14% for MI and 37% for micro-vascular complications. A recent Cochrane systematic review and meta-analysis summarized the impact of periodontal treatment on the glycemic control expressed by changes in Hb1A1c[351]. The authors included 35 parallel RCT accounting for a total number of 2565 participants. 94% of the trials were conducted on T2DM patients. The age range was

from 18 to 80 and the follow-up duration from 3 to 12 months. Data from 14 studies, 1499 participants, reported a beneficial effect of periodontal treatment on HbA1c with a mean percentage reduction of -0.29 (95% confidence interval (CI) -0.48 to -0.10; effect P = 0.003) at 3 or 4 months follow-up. Evidence from the 5 studies, 826 participants, describes no benefit of the periodontal intervention with mean percentage reduction in HbA1c of -0.02 (95% CI -0.20 to 0.16; effect P =0.84) at 6 months follow-up. However, the largest study included in the analysis with 514 participants and accounting for 20% of the total 2565 participants[352] has received a strong criticism by the scientific community for a several number of reasons[353]. Firstly, the HbA1c levels of the selected population were already close to the goal for a desirable glycaemia. Secondly, the clinical improvements in periodontal health following the treatment did not respect the acceptable standards of care. Finally, a high degree of obesity reported in the study population could have masked any change in the inflammatory response following the periodontal therapy.

1.7.1.2. Conclusions

For the time being, the evidence supporting the efficacy of periodontal treatment on the reduction of the hyperglycemia does not allow drawing final conclusions. The low quality of the trials and the lack of an adequate number of well-designed RCT indicate the need of further investigations.

1.7.2. Treatment of Periodontitis and CVD outcomes

1.7.2.1. Effects of Periodontal Therapy on CVD Morbidity

To date no randomized clinical trials have systematically investigated the role of therapeutic periodontal interventions in the prevention of cardiovascular events. Only two trials can be mentioned[354,355]. Paju et al examined 141 individuals with acute non-

Q-wave infarction or unstable angina pectoris in a double-blind, placebo controlled study with the use of clarithromycin for 3 months. The average follow-up period reported was of 519 days (1.4 years). The rationale of the study was based on the assumption that antibiotic therapy could impact on the progression of chronic infections including PD and therefore result in a reduction of CVD events rate. Antibiotic therapy however was not beneficial in prevention of recurrent cardiovascular events in patients with PD compared to healthy subjects. The second trial is a feasibility multicentre, randomized, controlled trial designed to study effects of periodontal intervention on CVD secondary prevention. Study participants were randomized to either community care or scaling and root planing in University settings. Despite being only a feasibility trial, after 1 year of follow up, no difference was observed in CVD events rate between the community control and the treatment groups (23 versus 24)[356]. As recently concluded by the American Heart Association position paper on the association between PD and CVD outcomes no evidence can be found in either favour or against it[164].

1.7.2.2. Effects of Periodontal Therapy on CVD Risk Factors

1.7.2.2.1. Traditional CVD Risk Factors

1.7.2.2.1.1. Lipids

Hyperlipidaemia is considered a well-established modifiable risk factor for coronary heart disease together with smoking, hypertension, glucose intolerance, obesity, and physical inactivity. Specific lipid biomarkers have been identified. Triglycerides (TG), serum total cholesterol (TC) and high/low-density lipoprotein cholesterol (HDL/LDL-C) are considered traditional lipid biomarkers associated with CVD whilst recent surrogate markers include Lp-PLA2 and oxidized LDL. Numerous studies have

demonstrated that lowering LDL-C levels can positively influence cardiovascular morbidity and mortality[357]. A meta-analysis reported that a 1.0 mmol/L reduction in LDL-C is associated with more than 20% reduction in CVD events[358]. Current guidelines refers to LDL-C concentrations that should be reduced to <2.6 mmol/L (100 mg/dL) in patients with established CVD and to <1.8– 2.0 mmol/L (70–80 mg/dL) in those with very high CVD risk[359]. Similarly reduction of TC to <4.5 mmol/L (174 mg/dL) is widely adopted as an effective strategy at reducing future CVD risk[359]. However, decreasing LDL-C levels to the recommended values, does not always abrogate the risk of having a major vascular event[358]. Epidemiologic studies have shown an inverse correlation between HDL-C and CVD events[360]. Low serum concentrations of HDL-C are an independent risk factor for CVD. Levels <1 mmol/L (40 mg/dL), increase substantially the risk for coronary heart disease[361,362]. Great attention has been given to changes in HDL-C as potential therapeutic targets for the treatment of CVD. Indeed HDL-c is an essential regulator of the reverse cholesterol transport pathway: a process that allows excess cholesterol stored in peripheral cells, such as foam cells, to be excreted by the liver via the bile. This process is believed to protect against atherosclerosis[363]. However, HDL-C has multiple additional protective properties including its role in the reduction of LDL oxidation. Oxidized LDL, easily absorbed by the macrophage, contributes to the formation of foam cells[364] and therefore impact on the progression of atheroma. HDL-C also decreases vascular inflammation[365], thrombosis, improve endothelial function[366], promotes endothelial repair[366] and increases insulin sensitivity[367]. HDL-C may also slow the progression of lesions by selectively reducing the production of endothelial cell–adhesion molecules that facilitate the uptake of cells into the vessel wall[368]. It therefore follows that increasing the concentration of HDL-C has the potential to reduce CVD risk. In addition to HDL/LDL close link to

atherogenesis, TG level is also considered an independent risk factor for CVD[357]. TGs are not directly atherogenic but represent an important biomarker of CVD risk because of their association with proatherogenic proteins[369]. Lp-PLA2 has been proved to be an independent risk factor for CVD[370] as it can hydrolyse oxidized LDL into pro-inflammatory mediators contributing to the atherogenic process. Two randomized controlled trials[371,372] examined a sample of otherwise healthy individuals affected by severe generalized PD and showed that non-surgical periodontal treatment caused reductions in total and LDL cholesterol levels at 2 and 6 months follow-up. In another RCT, Oz et al performed PD treatment in 50 individuals also suffering from hypercholesterolemia and evaluated their serum lipid concentrations 3 months after the treatment. There was a substantial decline in TC and LDL-C profiles in the treatment group and between the two groups[373]. Acharya et al described changes in TG and HDL-C, just after 2 months of PD therapy in subject with metabolic syndrome[374]. However, a number of clinical trials reporting no substantial differences in lipid profiles have also been found including individuals affected by PD[375,376]. Taylor reported a statistically significant difference in TC after PD intervention compared to control[377]. The most recent RCT reviewed, was conducted in China on a sample of 134 individuals with diabetes with a 6 months follow-up[378]. Participants were allocated to 3 different study arms; group 1 included patients undergoing non-surgical periodontal therapy at baseline and re-examined after 3 months, group 2 received treatment at 3 months follow up, and group 3 of no intervention. Serum lipid concentrations were reported as decreased in all groups but there were no statistical significant differences between the groups. Two clinical trials[379,380] reported changes in oxidized LDL after periodontal treatment. Montebugnoli et al showed a mean reduction in oxLDL of 18% in patients affected by PD and CHD whilst Tamaki demonstrated a 37% reduction

following PD therapy in otherwise healthy patients. In addition Losche et al found that the treatment of PD could influence the serum activity of Lp-PLA2[381]. Further reports were found on the effect of PD therapy on the structure and metabolism of HDL-C. In three prospective studies[382-384] HDL-C concentrations were raised 3 months after PD therapy and also authors demonstrated significant anti-atherogenic profile suggesting indirectly that PD could reduce the protective role of HDL-C on CVD onset. More than one third of the trials reviewed reported an improvement in serum lipid concentrations after PD therapy (reduction in total cholesterol and LDL in some and increase in HDL levels in other trials). This could represent a potential mechanism explaining the increased CVD risk in people with PD (i.e. worst cardio-metabolic profile of individuals diagnosed with PD). If these associations were proven causal then PD therapy could be suggested as additional approach to lipid lowering medications in further reducing CVD risk of the general population. However there is a wide variability among different trials outcomes reported. Factors such as age, gender, smoking status, general health status, medications, severity of periodontal disease and sample size should be taken into account comparing outcomes from different studies. Encouraging evidence supports the beneficial role of periodontal treatment and further large intervention trials assessing the potential additional effect of PD therapy in addition to lipid lowering medications should be performed.

1.7.2.2.2. Novel Cardiovascular Risk factors

1.7.2.2.2.1. Inflammatory markers

Inflammatory processes are recognized to play a central role in the pathogenesis of atherosclerosis and its complications[275,293]. Inflammation, focally and systemically, is involved in destabilization and rupture of atherosclerotic plaques, leading to acute

cardiovascular events[107]. Current risk prediction models based on traditional risk factors have the ability to predict long-term CV risk in many individuals. However great effort has been given to research novel risk factors to further improve CV risk prediction, with the aim also of detecting new targets for therapy and improve current prognostic algorithms. A large number of inflammatory markers have been studied in this context, and the list is constantly growing.

1.7.2.2.2.2. White blood cell count and differential

Circulating white blood cell (WBC) count represents a crude assessment of the individual inflammatory status[385] and has been proposed as a biomarker of CVD risk prediction[386]. A number of observational epidemiologic studies have reported a consistent positive association between WBC count and the risk of CVD[387-389].

Christgau et al in 1998 failed to show any differences in WBC in patients with diabetes and healthy controls 4 months after periodontal treatment[390]; these findings were confirmed in other trials[377,379,391-394]. In contrast, Christan et al. treated 27 PD patients showing that 3 months after therapy there was a significant reduction in WBC and that the effect of the intervention was more evident in non-smokers when compared to current smokers[395]. Similar results were obtained in two additional RCTs examining the effects of non-surgical intensive periodontal therapy on WBC after 6 months[12,372]. Lalla et al. showed a reduction in percentage of mononuclear cells (CD14+ monocytes) following periodontal treatment[396]. A recent report found that already 1 month after PD therapy individuals with CHD presented with a statistically significant reduction in WBC counts[397]. The efficacy of PD treatment was further confirmed in 3 additional clinical trials[398,399]. Based on the evidence examined, only 5 trials reported a statistically significant reduction of WBC following PD therapy whilst 9 other trials reported the opposite finding, we therefore conclude that there is inconclusive

evidence of a potential anti-inflammatory effect of periodontal therapy as assessed by raised WBC.

1.7.2.2.2.3. Acute-phase proteins

Acute-phase reactants are molecules produced during acute and chronic inflammation; they exert a variety of functions including activation of complement factors, neutralization of bacterial pathogens, stimulate repair and regeneration of a variety of tissues. These molecules are produced by the liver following a triggering stimulus (increased circulating cytokine levels) and have received great attention over the years and in particular C-reactive protein, plasminogen-activator 1 (PAI-1), and fibrinogen[400].

1.7.2.2.2.4. C-reactive protein

CRP is a protein mainly produced by the liver but also by adipocytes and vascular smooth muscle cells in response to a rise in interleukin-6 and tissue necrosis factor-alpha (TNF- α). It is a very stable and known acute-phase reactant[401]. CRP levels often increase substantially in response to a wide variety of biological insults, infections, inflammatory conditions and cancer[402]. However, given its consistent association with CVD, CRP remains an established marker of CVD risk, and it may very well be a contributor to the vascular inflammatory process in coronary arteries in humans. Multiple prospective cohort studies have established that increased CRP levels are associated with increased CVD risk in both genders, across a wide age range[403]. These findings have been consistent in different populations with diverse ethnic backgrounds and in diverse clinical settings, and CRP predicts a variety of CV outcomes, including incident AMI, stroke, sudden cardiac death, stroke, peripheral artery disease and also incident diabetes and new onset hypertension[404]. A recent meta-analysis showed that

serum CRP concentration has continuous associations with CVD risk, ischemic stroke and vascular mortality[405]. Risk ratio for CVD per 1-SD higher log(e) CRP concentration was 1.37 when adjusted for conventional risk. While CRP has multiple pro-inflammatory and pro-atherogenic properties, recent studies have not supported a causal role for it in atherogenesis[406]. CRP is primarily a non-specific marker of inflammation, and its levels rise in response to infections, autoimmune diseases and malignant processes. In the absence of inflammation, hsCRP levels of 1 mg/ml confer a lower risk for CVD, while levels above 3 mg/ml almost double the risk of CVD[407]. Multiple measures known to reduce CVD risk (i.e., smoking cessation, losing weight, exercise) also decrease serum CRP levels. Several medications, in particular, statins, are also known to reduce serum CRP levels[408]. The association between CRP and PD has been shown in several observational studies and at least 3 systematic reviews have examined the impact of PD therapy on CRP serum levels reduction. In the first meta-analysis, the weighted mean difference of CRP between cases with PD and controls was 1.56mg/l ($p<0.00001$)[409]. This data confirms that diagnosis of PD is associated with a state of low-grade systemic inflammation. In the same systematic review, data from six intervention studies was consistent with a 0.50 mg/L reduction of CRP serum levels after PD therapy (95% CI 0.08–0.93) ($p<0.02$). A second meta-analysis of intervention trials reported similar estimates with a mean overall difference in CRP serum levels after PD of 0.2 mg/L (95% CI -0.15-0.55)[410]. The most recent meta-analysis including 4 clinical trials reported a 0.23 mg/l reduction in CRP levels (-0.231; $p=0.000$)[411]. 2 additional trials performed in individuals with PD and other comorbidities including diabetes[378,412] confirmed the potential anti-inflammatory effect of PD therapy also in these populations. Sun et al, randomized 157 individuals with diabetes to a periodontal intervention or control. After three months, the serum level of CRP in the

treatment reduced from 5.81±1.23 mg/L to 5.51±1.29 with a mean difference of –0.30±0.31. Higashi et al, showed that six months after PD therapy individuals with CVD presented with a substantial reduction in CRP (from 2.7±1.9 to 1.8±0.9 mg/L) in the test group compared to the control[413]. Similar results were reported in individuals affected by PD and Metabolic Syndrome after eight weeks of PD therapy (reduction of 0.68 mg/L)[374]. Moderate evidence supports the notion that serum levels of CRP can be reduced by periodontal therapy. There is also sufficient evidence to suggest that periodontal therapy results in systemic inflammation as measured by CRP levels (of one week duration) and this perturbation lasts up to 1 month after the initial therapy session.

1.7.2.2.2.5. Fibrinogen

Substantial evidence has accumulated suggesting that fibrinogen represents a major risk factor for cardiovascular disease. Well-conducted meta-analyses have clearly shown that increased concentrations of fibrinogen are associated with the development or presence of atherothrombotic disease[414]. It was also reported[415] a strong association between fibrinogen and coronary artery calcification (CAC) and increased carotid intima–media thickness (CIMT), both being considered markers of subclinical coronary atherosclerosis[416]. Six studies reported a reduction whilst 2 reported an increase in fibrinogen levels following periodontal therapy. Bokhari et al confirmed a positive effect of non-surgical periodontal treatment in reducing fibrinogen levels in both patients with CVD or systemically healthy. Indeed individuals affected by CVD experienced a greater reduction in fibrinogen levels compared to healthy subjects[417]. Similarly Correa et al showed a significant decrease in fibrinogen levels in subjects with PD and type 2 diabetes 3 months after periodontal intervention[418] and Vidal et al in people with PD and hypertension[419]. However a larger

number of investigators were not able to replicate these findings[377,379,392,396,420,421]. Taylor et al showed a significant decrease of fibrinogen level 12 weeks after full-mouth tooth extraction[422]. The same group failed to replicate this finding in a further trial with a non-statistically significant reduction in fibrinogen profile in the treatment group compared with the control group[377]. Some controlled clinical studies show reductions in fibrinogen levels, but randomized clinical trials failed to demonstrate reductions in this marker. Two investigations confirmed that periodontal therapy results in the short-term increase of Fibrinogen levels. Thus, there is insufficient evidence to support fibrinogen as a biomarker or being affected by periodontal therapy.

1.7.2.2.2.6. SERUM AMYLOID A (SAA)

Serum amyloid A (SAA) is a systemic marker of acute and chronic inflammation. It also affects HDL composition and function[423]. SAA concentrations have been shown to positively correlate with the development of atherosclerosis[423]. SAA is raised in subjects affected by PD and comorbidity (i.e. CVD) however not in individuals with PD only[424]. Vuletic et al showed that 3 months after full mouth extraction levels of SAA were significantly reduced compared to pre-treatment[425]. Furthermore, Graziani et al reported first an increase and then a statistically significant reduction 6 months after periodontal therapy in a pilot study on 14 otherwise healthy patients with generalized advanced PD[391]. However Pussinen et al reported on 30 individuals otherwise health but suffering from PD who received PD therapy and reassessed 3 months after showed no reduction in SAA levels profile[384]. There is minimal evidence in support of a statistically significant effect of periodontal therapy on SAA (both in terms of increase or decrease).

1.7.2.2.2.7. Cytokines

Cytokines are small soluble proteins that transfer information from one cell to another. More than 200 cytokines have now been identified including interleukins, growth factors, chemokines, and interferons. They are all organized in complex networks playing fundamental roles in both pro/anti-inflammatory processes including CVD and PD. Several studies showed that compared to control subjects, patients with PD have higher concentrations of circulating inflammatory cytokines[426]. In addition, subjects with both CVD and PD show significantly higher concentration of cytokines compared to individuals with CVD only[413]. A bulk of evidence highlights the potential relevance of cytokines in mediating the inflammatory processes during atherogenesis and the development of CVD[427,428] and several studies investigated the efficacy of periodontal intervention on cytokine serum concentration.

1.7.2.2.2.8. Interleukins

Interleukins (ILs) are secreted proteins that bind to their specific receptors and play a role in the communication among leukocytes. ILs is a large family that comprises about 37 subgroups; among them the most studied because of their strong association with CVD are IL- 1, 6, 8, 10, 18. The pro-atherogenic effect of IL-1 is attributed to its ability to modulate a number of key events involved in the complex inflammatory process of atherogenesis such as the vessel wall inflammation, leukocyte chemotaxis and adhesion or plaque rupture[429-431]. Interleukin-6 (IL-6) is the main pro-coagulant cytokine[432]. It can increase plasma concentrations of fibrinogen and plasminogen activator inhibitor type-1 (PAI-1)[433], as well as increase the hepatic expression of CRP which amplifies a range of inflammatory and pro-coagulant responses[432]. IL-8 is a pro-inflammatory cytokine produced by various cell types involved in atherosclerosis, including endothelial cells, peripheral blood monocytes, and vascular smooth muscle

cells. The role of IL-8 in atherosclerosis could be mediated through its chemoattractant and mitogenic effects on vascular smooth muscle cells[434]. In addition, IL-8 plays an important role on the infiltration of monocytes into the sub-endothelial space, which is a crucial process in the early stages of atherosclerosis[435]. Serum levels of IL-10, a potent anti-inflammatory cytokine, have recently been shown to be reduced in patients with acute coronary syndromes[436], thus suggesting that may favour plaque instability and the development of acute coronary syndromes. Interleukin-18 (IL-18) is a pleiotropic pro-inflammatory cytokine which plays an important role in the inflammatory cascade[437] and potentially on atherosclerotic plaque progression and vulnerability[438]. Circulating concentrations of IL-18 have been prospectively associated with vascular events in patients with stable and unstable angina[439] or pre-existing CVD[440,441]. In 2 RCT from the same group, otherwise healthy individuals suffering from PD showed a reduction in IL-6 after PD therapy[372,442]. These results are in line with a subsequent report showing reductions in IL-6 levels in subjects affected by PD and CVD[413].Similar findings were shown in individuals suffering from PD and comorbidities like type 2 diabetes[412], hyperlipidaemia[443], refractory arterial hypertension[419]. Buhlin et al reported significant changes in IL-18 twelve months after periodontal therapy but not in other ILs (-1β,-4,-5,-6,-8,-10)[420]. We found also a number of trials which reported no efficacy of PD therapy on ILs profile[396,418,444]. The most consistent finding was a reported increase of IL-6 levels following periodontal therapy in the short term (1 month) and some evidence of a reduction in IL-6 levels in the medium terms (6 months). Therefore, we found moderate evidence of the impact of periodontal intervention on IL-6 and a less robust effect on other investigated IL

1.7.2.2.2.9. Tumor necrosis factor-α (TNF-α)

TNF-α is a significant independent predictor of CVD events and total mortality among men[445]. It is regarded as a pivotal pro-inflammatory cytokine but it can be detected in many human atheromas[446]. It is produced in murine and in human atherosclerotic lesions, primarily by macrophages/foam cells, activated T-cells, smooth muscle cells, and endothelial cells[447]. On a local level, TNF-α has the potential to promote cellular infiltration to the plaque via endothelial activation[448] and may induce endothelial dysfunction[449]. It also promotes the production of other cytokines as well as chemokine expression[450], the expression of matrix metalloproteinase-9[451], hence increasing plaque instability. TNF-α can also promote angiogenesis[452]. Individuals with PD showed higher TNF-α serum concentration compared to controls[426]. However we found only five trials reporting on the effect of PD therapy on serum levels of TNF-α. Iwamoto et al showed a significant reduction in TNF-α serum levels when comparing PD therapy and minocycline versus control[453,454]. These results are in line with other three clinical studies[412,418,455] on the effects of PD therapy on TNF-α in individuals with other comorbidities including diabetes. An equal number of trials failed to show a statistically significant effect of periodontal therapy on TNF- α[378,382,420,443,456]. Lastly Fokkema et al investigated the long-term effect of full-mouth tooth extraction therapy on the responsiveness of peripheral blood monocytes in a single subject with generalized terminal adult periodontitis reporting no changes in TNF-α release before and after therapy[398]. We conclude that there is some evidence on short term increase of TNF-α level following periodontal therapy but inconclusive evidence on the long term effects (reduction versus no effect).

1.7.2.2.2.10. CD40l/CD40

CD40 ligand is a member of the TNF superfamily, and interacts with CD40 (a TNF receptor superfamily member). Increased levels of sCD40L have been found in a range of chronic diseases including cardiovascular diseases, diabetes, peripheral arterial disease (PAD), pulmonary hypertension, acute/chronic heart failure, and stable/unstable angina[457,458]. CD40L has been investigated as a prospective risk marker of CVD[459]. Marcaccini et al reported the effects of periodontal therapy on CD40L levels in 20 controls and 25 patients with PD. At baseline, a statistically significant difference in the serum concentrations of CD40 ligand between the two groups (P = 0.009) was detected suggesting that the CD40 ligand may be increased in patients with periodontal disease. However no differences in CD40 ligand concentrations were found in the periodontal disease group (P = 0.41) 3 months after therapy[392]. We conclude that there is insufficient evidence on the effect of periodontal therapy on CD40 ligand.

1.7.2.2.2.11. Circulating cell adhesion molecules (CAMs)

Vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin are expressed by endothelial cells, macrophages and smooth muscle cells in response to various inflammatory stimuli (shear stress, oxidative stress, microbial stimulation or inflammatory mediators)[460]. CAMs are also involved in the adhesion and transmigration of leukocytes into the vascular endothelial wall thus promoting atheroma plaque growth and instability. Adhesion molecules are considered markers of vascular stress and their role in the pathogenesis of cardiovascular disease has been extensively investigated[107]. Selectins (CD62) promote transient rolling of leukocytes along the endothelium, whereas ICAM-1 (CD54) and VCAM-1 (CD106) mediate attachment and trans-endothelial migration of leucocytes[461].

CAMs, particularly sVCAm-1 are linked to future CVD death[462]. A number of studies indicate upregulated CAM expression at the gingival and systemic levels when PD is diagnosed[463]. The impact of periodontal treatment on CAMs expression/levels has also been investigated. We retrieved evidence from 6 different clinical trials. A statistically significant reduction of E-selectin level after PD therapy was reported in some of them[12,444,464]. No evidence of a similar effect of PD therapy on sICAM-1 and sVCAM-1 was found[392,396,444]. We found only one report with a reduction of sICAm-1 after PD therapy in patients with low CVD risk[465]. In summary we found some evidence of a short-term increase and medium-term reduction in s-Eselectin levels after periodontal therapy but not for other soluble cell adhesion molecules.

1.7.2.2.2.12. Monocyte chemoattractant protein-1 (MCP-1)

MCP-1, a member of the CC chemokine family, is involved in the pathogenesis of atherosclerosis by promoting recruitment of inflammatory cells to the vessel wall[466]. Serum MCP-1 concentrations have been found raised in individuals with periodontal diseases (1.5 folds in gingivitis, 3 folds higher in PD) when compared to healthy controls[467]. Further Fokkema et al reported a two-fold reduction of serum MCP-1 after dental clearance in a case report[398]. Despite this encouraging data, 3 clinical trials comparing serum MCP-1 concentration before and after PD therapy, failed to show any statistically significant reductions[392,396]. According to the current evidence, periodontal treatment seems to not be effective in the MCP-1 level reduction

1.7.2.2.2.13. Plasminogen Activator Inhibitor 1 (PAI-1)

Plasminogen activator inhibitor 1 (PAI-1) belongs to the family of serine protease inhibitors (SERPINs) and is produced in high quantity by a number of cells including endothelial cells in response to inflammatory cytokines. Raised PAI-1 plasma levels are

consistently found in individuals with severe sepsis but also with other acute or chronic inflammatory diseases such as atherosclerosis. PAI-1 is upregulated by inflammatory cytokines and may therefore be regarded as a marker for an ongoing inflammatory process[468]. Increased plasma levels of PAI-1 are positively correlated with the risk of developing CVD as well as with the extent of coronary sclerosis, restenosis, risk of myocardial infarction and deep vein thrombosis[469].

Montebugnoli et al[379] reported for the first time that individuals with PD presented with raised PAI-1 levels. Taylor et al showed significant reductions in PAI-1 3 months after full mouth extraction in people with terminal dentition due to PD[422]. However, the same group, in a latter study could not replicate the same findings[377]. Tonetti et al also confirmed that periodontal therapy did only increase acutely PAI-1 24 hours following periodontal therapy whilst no greater reductions were observed 6 months after PD therapy[12]. In addition, Lalla et al treated 10 diabetic patients affected by moderate/severe periodontitis and analysed PAI-1 levels 1 month after full mouth subgingival debridement. They showed no statistically significant difference[396]. Thus, there is currently no compelling evidence to support the effect of periodontal therapy on this marker although some evidence supports an increase rather than decrease of this marker in the short term.

1.7.2.2.2.14. D-DIMER

D-dimer is a marker of coagulation and specifically cross-linked fibrin turnover as derives from fibrin degradation. In coagulation disorders D-dimer profile can be severely altered with increased expression and linked to CVD[470-472]. D-dimer plasma levels are also considered a strong predictor of coronary disease[473]. PD therapy in 18 males aged 40–65 years suffering also from CVD did not produce a statistically significant reduction in D-dimer[379]. On the contrary two reports showed a significant

increase of D-dimer profiles 1 week after an intensive session of subgingival periodontal therapy and a subsequent decrease to baseline value within 1-3 month[391,464]. There is moderate evidence in support of an acute perturbation of periodontal therapy on d-dimers in the first month following treatment but no effect in the medium-long term.

1.7.2.2.2.15. VON WILLEBRAND FACTOR

Von Willebrand factor (vWF) is an endothelium-released glycoprotein[474]. It has been proposed as a valid biomarker of endothelial damage/dysfunction. Indeed raised plasma concentrations are often observed in inflammatory and atherosclerotic vascular diseases[458]. High plasma vWF levels (≥221 IU/dl) represent an independent risk factor for adverse CVD events[475]. Higher vWF antigen levels were reported in individuals with a poor dental status among individuals with acute myocardial infarction and healthy controls[146]. Furthermore, a more recent report confirmed increased vWF concentrations in individuals with severe PD when compared to healthy controls[476]. A small number of intervention trials reported no substantial reduction in vWF 3 months after PD therapy[377,379]. Two clinical trials showed instead a statistically significant increase of vWF levels within 1 month after intensive periodontal treatment[12,464]. There is insufficient evidence on the effects of periodontal therapy on vWF in the medium-long term, there is some evidence that periodontal therapy may increase vWF levels in the short term.

1.7.2.2.2.16. MMPs

The matrix metalloproteinases (MMPs) are a family of zinc-containing endo-proteinases that have all similar structural domains, but differ in terms of substrate specificity, cellular source, expression, and regulation. The metabolism of the

extracellular matrix is governed by a balance between the MMPs and their tissue inhibitors (TIMPs)[477]. Two of the MMPs biomarkers most consistently implicated in CVD development and prognosis are MMP-9 and TIMP-1[478]. Circulating levels of these MMPs have been related to most cardiovascular disease risk factors in large community-based samples[479] and have been associated with risk of death in patients with known CVD[480]. Our search identified 5 clinical trials reporting on the effect of periodontal therapy on MMPs. Higher serum circulating levels of MMP-3, -8, -9 were detected in patients with periodontal disease compared to healthy patients while no difference was found in MMP-2 and TIMP-1, -2[481]. In the same study, levels on MMPs were analysed 3 months after non-surgical PD treatment; a statistically significant difference was found in MMP- 8 and -9 while no effects were shown on MMP-2, -3 and TIMP-1, -2 levels (Table 7). Combined effects of PD therapy with adjunctive low-dose tetracycline therapy on MMPs have been shown in multiple studies[396,426,482]. Gorska et al compared conventional PD treatment alone or in combination with a low dose of doxycycline and showed a not statistically significant reduction in MMP-9 concentrations and TIMP-1 increase in both treatment groups[426]. Two further reports showed not significant trend in MMP-9 reduction[396,482]. Payne et al randomized 128 postmenopausal women with PD to either a twice-daily regimen of sub antimicrobial dose–doxycycline (SDD) or placebo tablets for two years as an adjunct to periodontal maintenance therapy. Their results were consistent with a reduction in MMP-9 in the SDD arm[483]. Insufficient evidence was found on the potential effect of periodontal therapy on MMPs levels.

1.7.2.2.2.17. Oxidative stress

Free radicals are highly reactive species characterized by an unpaired electron in their outer orbital[245]. They can damage proteins, lipids, carbohydrates and nucleic acids and

ultimately contribute to a number of pathogenetic processes in a variety of inflammatory disorders. Reactive oxygen species (ROS) include oxygen-derived free radicals, such as superoxide, hydroxyl, nitric oxide, hydrogen peroxide and hypochlorous acid[484,485]. Oxidative stress is defined as the condition arising from a serious imbalance between the levels of free radicals in a cell and its antioxidant defences in favour of the former[486]. When antioxidant systems are unable to counteract the free radicals action efficiently tissue damage ensues[487]. Oxidative stress has been implicated in a number of inflammatory diseases, such as type-2 diabetes[488], vascular diseases[489] and chronic inflammatory lung disease[490]. Oxidative stress does compromise endothelial cell function, a crucial mechanism in the development and progression of atherosclerosis[491]. In PD, excessive production of ROS has been demonstrated and appears as a result of local inflammatory responses[492-494]. Oxidative stress by-products including lipid peroxidation, protein carbonyl levels and antibodies against oxidized low-density lipoprotein are significantly elevated in individuals with PD when compared to healthy controls[336,495,496]. Furthermore, a significant difference in anti-oxidant capacity between individuals with PD and controls has been reported[380,497]. Montebugnoli et al described a significant decrease in Ox-LDL in 18 males with confirmed CCD 3 months after periodontal non-surgical therapy[379]. In a pilot study 14 otherwise healthy subjects with severe PD received a single session of intensive periodontal treatment showing a substantial increase of oxidative stress as assessed by circulating ROS with a progressive reduction up to 1 month[497]. These findings were confirmed by Tamaki et al who showed that improvement in periodontal parameters 2 months after non-surgical periodontal therapy was associated with a significant reduction in plasma ROM level in 19 systemically healthy subjects affected by PD[498]. In a second trial non-surgical periodontal treatment was also shown to be

produce a reduction in ox-LDL, and circulating oxidative stress in 22 otherwise healthy subjects with chronic periodontitis[380]. On the contrary Lalla et al[396] and Koromantzos et al[482] both failed to report a reduction of measures of oxidative stress in a individuals with PD and diabetes. In summary there is little comparative evidence on the role of periodontal therapy on biomarkers of oxidative stress, the available evidence suggests a possible reduction of these biomarkers in the medium term.

1.7.2.2.2.18. Blood pressure

Hypertension is widely recognized to play a key role in the development of CVD events such as cardiac and renal failure, stroke and myocardial infarction[499]. The definition of high blood pressure is defined by a systolic blood pressure over 140 mmHg and/or a diastolic blood pressure over 90mmHg in subjects who are not taking anti-hypertensive medication[500]. It has been hypothesized that the inflammatory chronic burden associated with PD could have hemodynamic influences and therefore impact on the pathogenesis and progression of hypertension[501]. Seinost et al reported no changes in blood pressure measures (diastolic/systolic) of 30 patients with severe PD, 3 months after periodontal intervention[393]. Conversely D'Aiuto et al reported a reduction of systolic blood pressure 2 months after intensive periodontal treatment in patients affected by severe generalized PD[372]. In a subsequent trial the same group failed to replicate these findings over a longer follow-up following non-surgical PD therapy[12]. Higashi et al in two RCTs reported not-significant effects of PD treatment on blood pressure[375,413]. Similar findings were reported in the remaining two trials examined[377,391]. There is moderate evidence supporting the ineffectiveness of periodontal therapy in reducing systolic and diastolic blood pressure.

1.7.2.2.2.19. Endothelial Function

The endothelium is involved in the regulation of vascular biology; anticoagulant and anti-inflammatory mechanisms, modulation of vascular growth and remodelling. The impairment of its functions is a key process in the early stage of the atherosclerosis and its progression[502]. Endothelial dysfunction can predict adverse CVD events and long-term outcomes[503]. Flow-mediated dilatation (FMD) represents the most widely used non-invasive ultrasound method to assess endothelial function measuring endothelium-dependent vasorelaxation of the brachial artery[504]. A case-control study showed that otherwise healthy subjects with severe PD had lower values of FMD compared to healthy controls[505]. PD therapy was consistently associated with a positive effect on endothelial function (improvement)[165,393,413,506-508]. Of the randomized studies the largest was Tonetti et al who randomized 121 healthy individuals suffering from PD to either a cycle of supragingival mechanical scaling and polishing (control) or full-mouth scaling and root planing, extraction of hopeless teeth and local delivery of microspheres of minocycline. Endothelial dependent function was significantly affected by periodontal therapy[12]. Higashi showed similar results in individuals suffering from hypertension and CVD but used different measures of endothelial dependent function[375,413]. In summary we report a consistent effect of periodontal therapy in improvement of endothelial dependent function. The studies examined provide moderate evidence that periodontal treatment has a positive effect on endothelial dependent function.

1.7.2.2.2.20. Subclinical atherosclerosis - Carotid Intima-Media Thickness (c-IMT)

Carotid-wall intima–media thickness is a surrogate measure of atherosclerosis[509] also associated with established CVD risk factors[510] and outcomes[511]. The intima–media thickness is the distance from the lumen–intima interface to the media–adventitia

interface of the artery wall, as measured by ultrasonographic images of the carotid arteries (not invasive). Evidence from observational studies suggests a moderate to strong association between PD and CIMT in otherwise healthy individuals[512-517]. Piconi et al[399] enrolled 35 otherwise healthy individuals affected by mild to moderate PD in a RCT. Participants received a scan of their carotids before and after PD therapy. An intragroup reduction of CIMT at 6 and 12 months after the periodontal intervention was reported. Insufficient evidence was found on the possible effect of periodontal therapy on the rate of progression of CIMT.

1.7.2.3. Conclusions

After more than 30 years from the first reports on the association between dental infections/periodontitis and CVD outcomes, we are still debating on whether these associations are causal or casual in nature. Over the last 10 years the number of clinical intervention trials has multiplied including both traditional and novel CVD outcomes in response to periodontal therapy. However after a critical appraisal of the evidence reported to date we confirm that there is still minimal or insufficient comparative evidence on the effect of periodontal therapy on CVD outcomes and or subclinical atherosclerosis. Nevertheless a number of clinical trials have helped in our understanding of the potential systemic implications of periodontal therapy. Indeed this therapy is strongly associated with a host response which also seems to be modulated in a time dependent fashion. Periodontal therapy which often consists in the mechanical cleaning of the PD diseased dentition is often associated with a local and systemic amplification of the body inflammatory response. An acute inflammatory response (characterized by sharp increase in a number of inflammatory biomarkers including, CRP, IL-6, TNF-α) has been consistently reported by several investigators. This inflammatory state is also associated with a perturbation of the haemostatic

system (fibrinogen, d-dimers, PAI-1) and a state of endothelial cell activation (s-Eselectin, vWF) and impairment of endothelial function (as assessed by flow mediated dilatation of the brachial artery). The systemic implications of these acute effects are not fully understood at this time. This is why we suggest that further investigations should be performed in further understanding this area of research especially in high risk populations including individuals with associated co-morbidities (i.e. diabetes mellitus or diagnosed CVD). There is already a wealth of evidence suggesting that episodes of acute infection/inflammation are associated with a short term increased vascular risk[518] and one report suggests that this finding could also be true in individuals undergoing simple dental treatment procedures like a tooth extraction[519]. Following 1-2 months from periodontal therapy, all comparative evidence retrieved by the authors is suggestive of a progressive reduction/improvement in traditional (lipid markers) and novel (CRP, IL-6, fibrinogen, s-Eselectin) CVD risk factors. Interpreting this data with caution could represent the basis for inclusion of periodontal assessment and therapy as the key determinants in controlling each individual CVD risk. However a number of flaws were identified when appraising the available evidence on the effect of periodontal therapy on CVD outcomes. Firstly the majority of clinical trials is of limited sample size (<500) and the length of follow-up limited to 6-12 months. This represents perhaps the most important limitation in interpreting the data. Both PD and CVD represent long-term chronic conditions. In particular atheroma formation has been identified very early in life (i.e. already in young children). We could speculate therefore that performing some periodontal treatment only at the end of atheroma evolution (much later in life) might not represent an effective method of preventing further progression of the disease nor the occurrence of acute vascular events. Further research efforts should be devoted in designed appropriate clinical trials on the

delivery of effective oral health promotion early in life and monitor the potential beneficial effects on systemic health much later in life. Further, the multifactorial aetiology of both PD and CVD and the fact that both share a common inflammatory nature would indicate that the mere control/removal of local gingival infection might not be sufficient in producing a systemic sustained benefit. In turn additional therapeutic approaches should be researched including host modulation therapies in combination with standard periodontal therapy. These novel approaches at this time have not been fully tested also because of the limited knowledge of the mechanisms involved in the onset and progression of both PD and CVD. The only consistent effect of periodontal therapy was found on measures of endothelial function that represents a surrogate marker of CVD. Insufficient evidence was found on the effect of periodontal therapy on c-IMT and other measures of subclinical atherosclerosis. Whilst endothelial dysfunction is predictive of future CVD risk/outcomes, it is also a research measure greatly confounded by a number of methodological and environmental factors and therefore does not represent an efficient research outcome to be implemented in large scale intervention trials.

1.7.2.4. Conclusions

In conclusion large scale/multinational intervention trials have not been conducted on the effect of periodontal therapy on traditional or non-traditional CVD risk factors. Further and more importantly most of the clinical trials were performed in university/hospital settings that perhaps would not well represent the every-day periodontal clinical care.

1.8. AIMS

Our groups has previously shown that periodontitis is associated with a reversible state of systemic inflammation[303] oxidative stress[497] and endothelial dysfunction[12]. Indeed an intensive session of periodontal therapy induced first an acute vascular dysfunction and increased oxidative stress, but later a resolution of these processes with an improved vascular function[12].

The specific aims of this project were to:

1) To quantify the magnitude of association between periodontitis and surrogate markers of endothelial dysfunction and subclinical atherosclerosis.

2) To investigate the role of periodontal treatment on systemic inflammation, oxidative stress, endothelial function and metabolic control in patients with PD and T2DM.

3) To investigate the role of chronic inflammation related to periodontitis on endothelium integrity in patients with PD and T2DM.

4) To explore potential mechanisms linking acute inflammation with endothelial dysfunction in patients receiving periodontal therapy.

1.9. RESEARCH HYPOTHESIS

Based on the research question, aim and objectives, the overall main hypothesis of the research programme was “to investigate the null hypothesis of no association between periodontitis and its treatment with vascular dysfunction”.

Specific hypotheses:

Study 1: To test the null hypothesis of no association between periodontitis and its treatment, flow-mediated dilatation and carotid intima-media thickness.

Study 2: To test the null hypothesis of no between groups difference in flow mediated dilatation 6 months after periodontal treatment compared to control therapy in patients suffering from moderate to severe periodontitis.

Study 3: To test the null hypothesis of no between groups difference in carotid intima-media thickness 12 months after intensive periodontal treatment compared to control therapy in patients suffering from moderate to severe periodontitis

Study 4: To test the null hypothesis of no between groups difference in flow mediated dilatation 24hrs after a single session of non-surgical periodontal treatment in patients who received remote ischemic preconditioning 30 minutes before the intervention compared to placebo.

2. Methods

2.1. Periodontal Assessment

Clinical periodontal parameters were performed by a single calibrated examiner using a manual University of North Carolina (UNC-15) periodontal probe at six sites per tooth (i.e., mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, and disto-lingual) on all teeth (excluding 3rd molars). At proximal sites (mesio-buccal, disto-buccal, mesio-lingual, disto-lingual), the probe tip was placed as close to the interproximal area as possible. On the buccal and lingual surfaces of the tooth, measurements were made at the mid-buccal, mid-lingual points and the probe followed the root contour. Whenever a clinical measure was between two millimetres marks of the periodontal probe it was rounded up. With the periodontal probe in position and after calling the pocket depth reading, the examiner recorded the recession (REC) as a positive or negative value if the free gingival margin (FGM) occurred coronal or apical respectively to the cemento-enamel junction (CEJ). In the latter case the examiner reinserted the probe angled 45° degrees into the site in order to detect the CEJ. If the CEJ was not detectable for anatomical or restorative reasons the examiner adopted clinical landmarks as follow:

- Crown Margin (Recorded as Cr)
- Rotated teeth, mid-buccal and mid-lingual readings were taken from the clinical midpoints

Clinical attachment level (CAL) was calculated from the formula PPD minus REC. Full mouth plaque scores (FMPS) was recorded as the percentages of total surfaces (6 aspects per tooth), which revealed the presence of plaque. A binary score was assigned to each surface (1 for plaque present, 0 for absent)[520]. A full mouth bleeding score was recorded in the same manner.

2.2. Periodontal Treatment

Individuals included into the study 2 and 3 were randomized to two different periodontal therapy regimens: standard (CPT) or intensive (IPT) periodontal therapy. Patients in the IPT group received an intensive treatment consisting of mechanical debridement with ultrasonic and hand instruments of the diseased dentition in a single session (2-3 hours). Any tooth that from the baseline examination was defined as hopeless or irrational to treat was extracted during the treatment session. Local anaesthesia was used as necessary. Control group patients (CPT) received a standard cycle of periodontal therapy consisting of oral hygiene instructions, supra-gingival mechanical instrumentation and polishing in one appointment performed as appropriate by a single clinician using a combination of hand and machine driven (piezoelectric) instrumentation. After initial periodontal intervention, participants in the IPT and CPT groups were re-evaluated at 2 months and received additional therapy planned accordingly. For the IPT group a Modified Widman Surgical Flap procedure was performed in all areas with remaining periodontal infection (PPD>4mm and bleeding on probing) as previously described for those individuals with good oral hygiene (dental plaque scores less than 25%)[521]. The goals of this corrective therapy were to: 1) gain access for root preparation when non-surgical methods were ineffective; 2) establish favorable gingival contours; 3) facilitate oral hygiene. Those individuals in the IPT group who presented with suboptimal oral hygiene received additional sessions of non surgical periodontal therapy (whole mouth). CPT received a full mouth scaling and polishing. Supportive periodontal therapy, formerly referred to as periodontal maintenance including an update of the medical and dental histories, examination of extra- and intraoral soft tissues, dental examination, evaluation of the patient's oral hygiene performance and supra- and sub-gingival removal of bacterial

plaque and calculus according to group allocation was performed after 6 months follow up in Study 3. The therapeutic goals of SPT were to: i) prevent or minimize the recurrence and progression of periodontal disease, ii) prevent or reduce the incidence of tooth loss. IPT group received a single session of full mouth scaling and root planing while a single session of scaling and polishing was performed on CPT group. In both Study 2 and 3, upon observation of progression of periodontitis, defined using the EFP criteria[522], at anytime during the study they were exited from the investigation and received appropriate specialist care according to the UCL Eastman Dental Institute guidelines. At the end of the study, all patients who had received CPT, were provided with IPT therapy as required. Individuals included into the study 4 were allocated to a single session of IPT treatment.

2.3. Blood Sampling

Blood samples were collected by single venepuncture from patient's arm complying with the standard procedure of the local hospital. 1 8 ml silicone coated glass tube, 1 6 ml Spray-coated K_2EDTA and 2 4ml Buffered sodium citrate 0.105 M (≈3.2%) glass tubes were collected at every visit and processed for serum and plasma separation for Study 2,3 and 4. Silicone coated glass tubes were centrifuged at 4000 RPM for 15 minutes within 1 hour from the collection. EDTA and Citrate tubes were gently inverted for few times and centrifuged at 4000 RPM for 15 minutes straight after the collection. Serum and plasma were subsequently stored in multiple aliquots in a -70° freezer. In study 4 an additional 20 ml sample of heparinised blood was taken for the real time isolation of peripheral blood mononuclear cells (PBMC) and oxidative status assessment.

2.4. Carotid Intima Media Thickness (c-IMT) as a CV Risk Predictor

The assessment of the intima-media thickness of the carotid artery (c-IMT) by B mode ultrasound is a tool introduced to evaluate the presence and progression of atherosclerosis and estimate future CV risk. Being a non-invasive, safe and well tolerated exam contributed to its adoption in both clinical and research settings. Pignoli et al. in 1986 reported a good correlation between histology and ultrasound assessment even if the latter could provide with a minimally higher measure of c-IMT[523]. The interest in c-IMT measurements derives from multiple observational studies. 1,257 male subjects of the Kuopio Ischemic Heart Disease Risk Factor (KIHD) study were followed for 2 years correlating c-IMT with the risk of myocardial infarction. There was an increased relative risk of 4.1 (95% confidence interval [CI] 1.8 to 9.2) in the presence of a plaque and if c-IMT was>1 mm there was a 2.1 fold risk of MI. Using c-IMT as a continuous variable led to 11% higher risk of MI each 0.1mm increase in c-IMT[524]. The Atherosclerosis Risk in Communities (ARIC) study observed a population of adults with no history of disease for 4 to 7 years. Having c-IMT > 1mm was related to a hazard ratio of 2.62 (95% CI 1.55 to 4.46) in women and 1.20 (95% CI 0.81 to 1.77) in men after adjusting for CV risk factors[525]. However, the Rotterdam study reported a similar CV risk between genders[526]. Additionally, the investigators of the Carotid Intima Media Thickness [IMT] and IMT-Progression as Predictors of Vascular Events in a High Risk European Population (IMPROVE) study indicated that other parameters related to the common carotids, such as the increased intra-adventitia diameter can be predictors of CV events independently from the Framingham risk score[527]. c-IMT has also been associated with classic CV risk factors such as diabetes and hypercholesterolemia[528-530]. Hypertension is related to an increased c-IMT possibly due to a medial hypertrophy[531,532]. Interestingly,

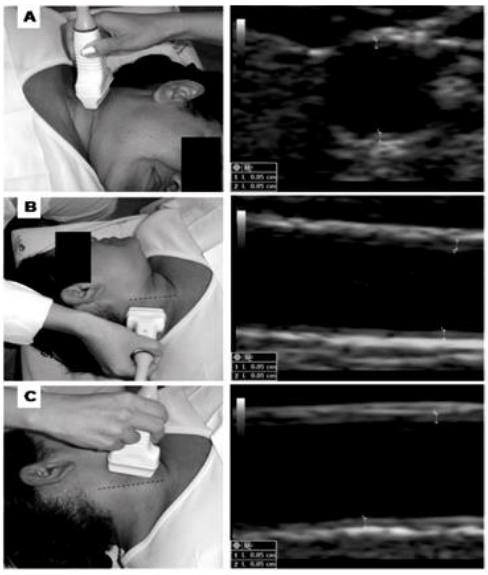
pharmaceutical interventions seem to have an impact on c-IMT. Blood pressure and lipid lowering agents can reduce the progression of c-IMT or decrease its value[533-536]. Bogalusa Heart Study reported the presence association between c-IMT and CVD since young age[537]. The Pravastatin, Lipids, and Atherosclerosis in the Carotid Arteries II (PLAC-II) study reported 0.0295 instead of 0.0456 mm/year of progression between test and controls[538]. Rosuvastatin in the The Measuring Effects on Intima-Media Thickness: An Evaluation of Rosuvastatin (METEOR) study showed changes of −0.0014 and 0.0131 mm/year comparing test and placebo in a middle-aged populations with low Framingham risk scores (FRS)[539]. In addition the Monitored Atherosclerosis Regression Study suggested that changes in lifestyle such as smoking cessation and weight loss are associated with a 0.13mm/year reduction in progression of c-IMT. The progression rate ranges from 0.006mm/year in healthy participants to 0.06mm/year in patients with coronary artery disease[540]. In a post-mortem study, the increased aortic wall was related to mainly intimal changes. c-IMT augments 2 to 3 fold during life, however gender and ethnicity seems to partially explain a certain heterogeneity showing the highest values in Afro-Caribbean population and the lowest in Hispanics[541].

2.4.1. METHODOLOGY

c-IMT can be assessed with different ultrasound techniques. M-mode ultrasound provides with a higher temporal resolution but can assess only a single point rather than a segment. The thickening of the carotid is not uniform therefore a single point measurement could cause a loss of important information. In addition, in M-mode, the estimate of a dimensional image is related to multiples of the pixel size increasing potential errors since the thickening of the vascular walls could be of a sub-pixel magnitude. c-IMT is normally an average measure of the values detected during the

cardiac cycle, its most used value is relative to the end-diastole since there is a lumen expansion observed during systolic phase. There is still no agreement on which segment should be selected for the c-IMT evaluation. The most used is the common carotid artery (CCA) meanwhile both internal (ICA) and carotid bulb measurements are less common[542]. The convenience of CCA is its easier imaging compared to ICA[543]. The IMPROVE trial reported an equal strength of association to CV risk for all the segments even if atherosclerosis starts earlier in ICA or the carotid bulb[527]. IMT could be assessed also in other arterial walls such as the brachial, radial or femoral arteries. However changes in CCA seems to be mainly dependent on intimal thickening meanwhile for other sites the tunica media has an equal contribution to the modification. The far wall of CCA is preferred to the near wall measure since it shows a higher correspondence with histologic values[544]. Therefore the current protocol recommended far wall images of the segment 1-2 cm distal to the carotid bulb. Following international guidelines the scanning protocol suggest to have the subject in a supine position with a light hyperextension and rotation of the neck to the contralateral side (Figure 11).

Figure 11 c-IMT acquisition technique



The subject lay supine with slight hyperextension and rotation of the neck to the contralateral side of acquisition. The sonographer (positioned on top of the bed, behind the head of the patients) places a high resolution ultrasound probe on the middle of the patient neck. The proximal part of the carotid bulb was identified in transversal plane. This allowed identification the region of interest of the common carotid artery, 1cm below the bulb. B) With slow movements of the probe, the longitudinal axis of the artery was visualised. C) Images were optimised to obtain the best view of the

intima-media thickness of the far arterial wall. The optimal longitudinal image was acquired continuously for 10 seconds and video-clips were stored for post-acquisition analysis.

Right and left carotid arteries were detected with a linear-array transducer at 12 MHz frequencies connected to a high-resolution sonograph. All the measurements were then analysed offline with an echo-tracking package that provides a semi-automated border detection system.

2.5. Flow-Mediated Dilatation (FMD)

The endothelium contributes to the regulation of the vessel tone therefore its functionality has an impact on this process[502]. In 1980s experiments stimulating isolated rabbit aortic blood vessels with acetylcholine showed vaso-relaxation subject to the integrity of the endothelium and mediated by nitric oxide (NO)[545]. The administration of acetylcholine and other pharmacological agents such as adenosine and papaverine can increase the blood flow[546]. In 1992, flow-mediated dilatation (FMD) a non-invasive method was introduced to assess the endothelial regulation of the vessel tone in conduit arteries[547]. FMD is based on the high-resolution ultrasound evaluation of the dilation of the brachial artery subsequent to an increased blood flow obtained with the inflation and release of a sphygmomanometer cuff on the forearm. Inhibitors of NO can abolish this mechanism suggesting its dependence on NO bioavailability[548]. Impaired FMD is associated to different CV risk factors and it can improve after treatment[549]. In addition, endothelial dysfunction of the brachial artery is linked to the coronary arteries endothelial function suggesting its relation with the development and progression of atherosclerosis[550]. FMD therefore is a non-invasive method to study the vascular homeostasis in subjects with no clinical signs of CVD[551].

2.5.1. METHODOLOGY

To be a reliable measure, FMD requires standardization in both image acquisition and analysis. In addition there are some individuals' related factors that could increase the variability of the measurements such as mental stress, caffeine, smoking, medications, menstrual cycle and room temperature[552,553]. The procedure performed in our studies, required 8 to 12 hours fasting and to be performed in a temperature controlled room[554]. Tobacco usage should be avoided for 4 to 6 hours[555]. However the importance of environmental factors has been recently reconsidered. The protocol required the participants to rest 10 min before the procedure[556]. The brachial artery represented the preferred area to evaluate since vessel with a smaller diameter reduce the accuracy of the reproduction and also variations in the absolute diameter would be reflected in large percentage changes. Once the subject was lying supine with the arm placed on a specific armrest, a high-resolution probe detected longitudinal images of the brachial artery. The measurements of the brachial diameter were obtained at the end-diastole to avoid vessel changes during the systole[557]. In order to insure reproducibility at different time points the position of the arm, the distance between the probe and the pneumatic cuff together with a thermal print of the arterial image were recorded. A blood pressure cuff was placed on the forearm within 2 cm from the antecubital fossa in order to obtain a higher reactive hyperaemia[558]. Positioning the cuff proximally can also affect the peak flow and reduce the accuracy of the imaging since the artery can collapse during the cuff inflation. To trigger a sufficient hyperaemia the cuff occlusion time is 5 minutes and evidence reported a non-significant difference in the dilation if the time is increased to 10 min[553]. In order to reach ischemia the pressure inflated was 50 mmHG higher than the systolic pressure. Once the cuff was released, a reactive hyperaemia in the brachial

artery triggered the vessel dilatation explicated by a functional endothelium. The vessel diameter reached its peak 45-60 seconds after the cuff deflation. A semi-automatic software allowed to evaluate the changes in the brachial artery diameter during the hyperaemic flow. FMD was reported as the change in percentage of the vessel diameter compared to its baseline value. It is important to consider the influence of the vessel diameter on FMD since the size has an impact on the blood flow shear stress therefore it is included in its calculation. The absolute change expressed in mm is not related the diameter therefore for completeness the results should report the baseline diameter, the absolute and the percentage changes.

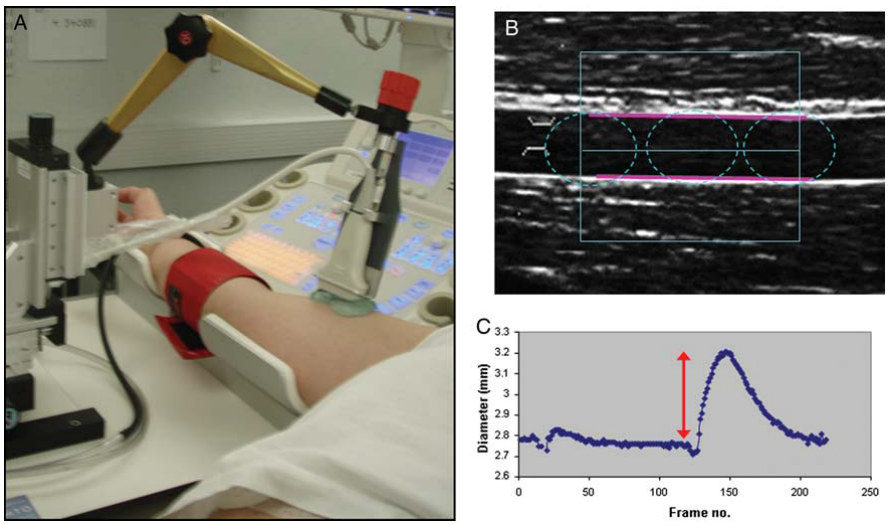


Figure 12 FMD flow-mediated dilatation settings

(A) Advised positioning of the sphygmomanometer cuff and the ultrasound probe. (B) Identification of the region of interest using the edge detection analysis software FMD analysis. (C) Output generated by edge detection software that assess the vessel dilation.

After 10 minutes of rest, endothelium-independent dilatation was measured after sublingual administration of 25 µg of nitroglycerin, according to the same recording protocol.

2.6. PBMC isolation by density gradient centrifugation

PBMCs were isolated using density gradient media (Ficoll-Paque PLUS). 15ml of blood collected in preservative free heparin was mixed with 15ml RPMI 1640 containing L-glutamine with added antibiotics (penicillin 100U/ml and streptomycin 100mg/ml) and layered over 20ml of Ficoll-Paque PLUS in a 50ml conical tube. The sample was then centrifuged at 22°C for 25 minutes at 700g with no brake. A wide tipped Pasteur pipette was used to aspirate PBMCs from the buffy coat layer formed (Figure 13) into a fresh 50ml conical tube,

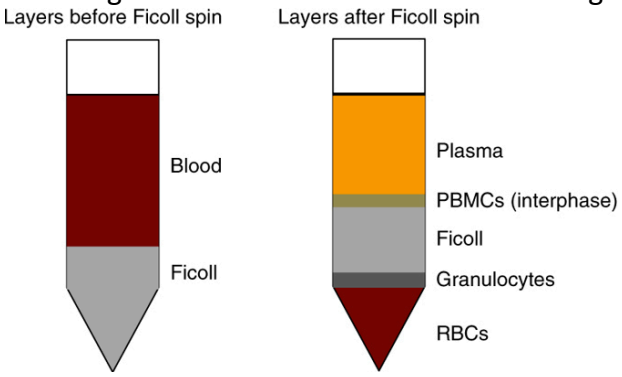


Figure 13 PBMC gradient isolation

which was then washed with RPMI 1640 containing L-glutamine (with 10% heat inactivated FCS and antibiotics as before). Cells were centrifuged at 500g for 10 minutes and the pellet re-suspended in RPMI 1640 (with FCS and antibiotics). Cells were counted as described previously. To allow batch analysis aliquots of cells (concentration 5-10x10^6 cells/ml calculated with a hemocytometer) were re-suspended in heat inactivated fetal bovine serum with 10% DMSO and frozen in a freezing container placed in a -80°C freezer for 15-24 hours and then submerged in liquid nitrogen for use in later experiments.

2.7. Measurements of Oxidative Stress

The majority of the methods available for the evaluation of oxidative stress *in vivo* are not suitable for large-scale use, limiting their application in clinical practice[559-561]. In addition, selecting one specific biomarker as representative of the total oxidative

stress or anti-oxidant state in specific patient categories is still debatable. Indeed, each disease can be characterized by increased production of specific oxidative species, depending on their intracellular sources. Electron spin resonance (ESR) is currently considered the gold standard assay to measure different RS in biological samples[562], however this technique is complex and not available in most clinical laboratories. Furthermore, ESR uses different protocols depending on which oxidative species are targeted, requiring continuous adaptation of the experiment and machine parameters and introducing further complexity in the measurements. Ideally, the perfect oxidative stress assay should be easy to perform, reliable, quick and inexpensive. Automated analysers would allow processing of a large number of samples, avoiding manual sample and reagent handling, and reducing variability sources. Among the various commercially available kits, the reactive oxygen metabolites (dROM) test have been developed to assess in an automated, economic and reproducible fashion the total amount of oxidant species in different biological samples.

2.7.1. d-ROM Test

The d-ROMs test measures the blood concentration of hydroperoxides, a class of chemical oxidant species belonging to the wider group of reactive oxygen metabolites[563,564]. Hydroperoxides are generated by the oxidation of several molecules such as glucosides, lipids, amino acids, peptides, proteins, and nucleotides, making their levels independent of the source of oxidative stress. In the presence of free iron, hydroperoxides are able to generate alkoxyl and peroxyl radicals, according to the Fenton's reaction[565,566]:

$ROOH + Fe^{2+} \rightarrow RO^{*} + OH^{-} + Fe^{3+}$

$ROOH + Fe^{3+} \rightarrow ROO^{*} + H^{+} + Fe^{2+}$

Such radicals are highly reactive and can quickly oxidise surrounding positively charged molecules, in order to recover their electron stability. By adding to the solution a stable and positively charged radical (N,N-diethylparaphenilendiamide radical) which rapidly interact with the alkoxyl and peroxyl radicals becoming pink after oxidation, the d-ROM test photometrically measures the amount of alkoxyl and peroxyl radicals formed by degradation of hydroperoxides (Fig. 14).

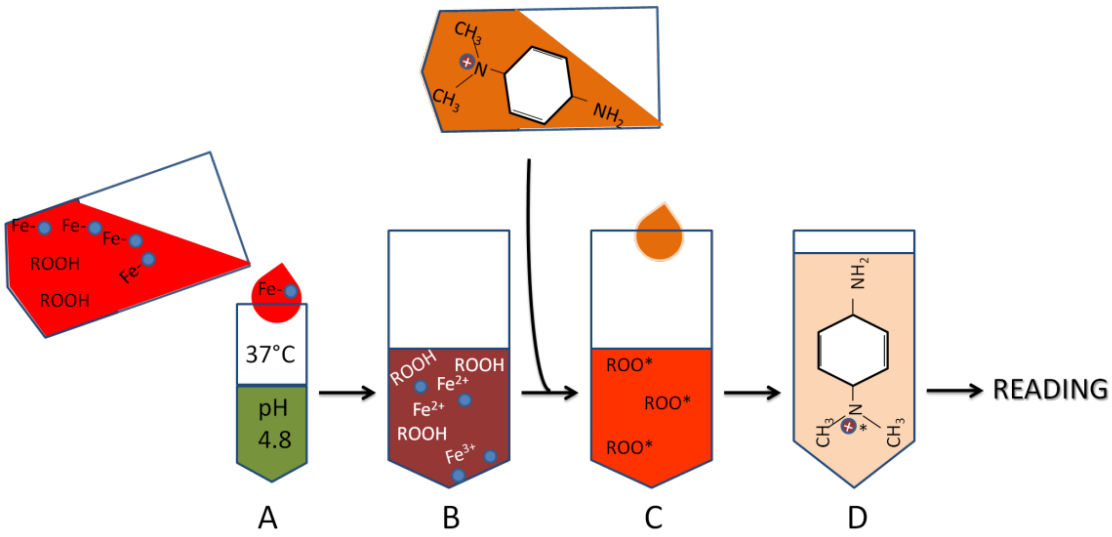


Figure 14 d-ROM Test

The reaction is starts by adding a small amount of serum to a solution containing N,N-diethylparaphenilendiamide radical and an acidic buffer (pH 4.8). The solution is warmed up to 37°C. This detaches the iron ions from serum protein, making them available for the Fenton reaction and transforming the hydorperoxides in the samples in alkoxyl and peroxyl radicals. The latters rapidly react with the N,N-diethylparaphenilendiamide radical, causing a change in the colour of the solution which shifts to pink. The intensity of the pink colour is directly related to the original amount of hydroperoxides in the sample and is readable using a photometer with absorbance at 505 nm.

2.7.1.1. Assay procedure

20µL of serum are diluted in a solution formed by: a) 10µL of a chromogenic mixture containing 10% of the positively charged N,N-diethylparaphenilendiamide radical and b) 1ml of a buffer containing HCl with pH 4.8 (Figure 13 A and B). The sample is warmed at 37°C for 1 minute. The combination of the high temperature and acidic environment allows detachment of iron ions from serum proteins, making them available for the Fenton reaction (Figure 14 B and C). Thus, the hydorperoxides in the samples are transformed in alkoxyl and peroxyl radicals which rapidly react and oxidize the N,N-diethylparaphenilendiamide (Figure 14 C and D). This determines a progressive change in the colour of the solution (towards pink) which can be read by a photometer (absorbance at 505 nm) (Figure 14 D). The final reading is dynamic and is performed immediately as well as at 1, 2, and 3 min following incubation. Obviously, the level of absorbance is directly dependent on the amount of the reactive oxygen metabolites present in the serum, according to the Lambert–Beer's law. A blank reagent obtained by replacing serum with distilled water and a standard with assigned value are included for each series of assays. The results of d-ROM test are expressed in arbitrary units called “Carratelli Units” (CARR U), according to the following formula:

CARR U = F(ΔAbs/min) where *F* is a correction factor (approximately 9000 at 37°C according to the results obtained with the standard); (ΔAbs/min) are the mean differences of the absorbances recorded at 1, 2, and 3 min. Reference values of healthy subjects are between 250 and 300 CARR U; conditions of slight, medium, and high oxidative stress are defined, respectively, by values of 320–360, 360–400, and >400 CARR U; for values up to 500 CARR U, a sample dilution is required[567]. Results obtained with d-ROM test have been validated by electronic spin resonance spectroscopy and it has been experimentally established that 1 CARR U corresponds to

0.08 mg of H_2O_2/dl[567]. Furthermore, it has been proved that, in vertebrate blood samples, results are stable also after refrigeration of the sample.

In this study, the intra-assay coefficient of variation obtained by running 20 randomly selected samples in duplicate was 3%. All analyses were performed in a blind fashion.

2.7.2. Flow cytometry for detection of mitochondrial oxidative stress and membrane potential

Peripheral blood mononuclear cells (PBMC) were isolated following standard procedures by density gradient centrifugation with Ficoll (Ficoll-Paque PLUS, GE, UK) from an aliquot of heparinised blood collected at each study visit. Mitochondrial oxidative stress production and membrane potential were assessed by flow cytometry using the mito probe MitoSOX Red (Invitrogen, UK) and 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen), respectively.

2.7.2.1. JC-1

For JC-1 staining, we followed the instructions recommended in the kit, which have been previously validated for PBMC staining[568]. PBMC suspension was adjusted to a concentration of 1 x 10^6 cells/mL and incubated in phenol red free RPMI 1640 with JC-1 (concentration 2uM) for 30 min at 37°C and 5% CO_2 in the dark. Cells were washed three times with warm phosphate-buffered saline (PBS) (PAA Laboratories), resuspended in 5 mL of warm RPMI and immediately analysed using a FACScan flow cytometer (Becton Dickinson). Data were acquired using CellQuest software version 3.1f (Becton Dickinson) and post-acquisition analysis was performed using FlowJo (FlowJo LLC, Oregon, USA). The mitochondrial uncoupling agent CCCP (carbonyl cyanide 3-chlorophenylhydrazone, Invitrogen) was used as positive control for each measurement. As CCCP completely depolarizes the mitochondria of all cells[569], it

enables correction for JC-1 aspecific staining e.g., dye loading of the cells. CCCP was added to the samples at a final concentration of 50µm, 5 minutes before adding JC-1, as per manufacturer protocol. In each sample (with and without CCCP), the mitochondrial membrane potential was estimated from the ratio of the median fluorescence value for red (FL-2) divided by the median fluorescence value for green (FL-1). Finally, the ratio obtained from the JC-1 sample without CCCP was divided for the ratio obtained from the sample with CCCP to exclude misreading due to aspecific staining of the JC-1[568,570]. Previous studies demonstrated a good reproducibility of the assay using PBMC isolated from subjects with severe inflammatory diseases[568]. This is confirmed from our preliminary data. Indeed, using PBMC isolated from 2 healthy subjects in 2 different days we found an inter-assay coefficient of variation of 8%.

2.7.2.2. Mitosox

Another aliquot of 2x 10^6 PBMC were used for the estimation of the mROS production using MitoSOX™ Red mitochondrial superoxide indicator. This probe is highly selective for mitochondria superoxide detection is considered the gold standard probe for fluorescent detection of mROS in live cells, including PMBC [262,263,571]. We used kit instructions and a previously validated protocol to assess mtROS production[572]. PBMC were re-suspended in 1ml of phenol red free RPMI and incubated for 20 minutes at 37°C, in the dark and in a 5% CO2 incubator with MitoSOX™ Red reagent (final concentration of 5 µM). After three washing steps with warm buffer (37°C), cells were immediately analysed using the same flow cytometer and software used for the JC-1 assay. The value of median intensity fluorescence in FL2 was used as average amount of the mitochondrial superoxide production. Mitochondrial membrane potential and superoxide production was assessed in the whole PBMC population as well as in the

lymphocyte and monocyte subpopulations separately identified by forward and side scatter characteristics.

In each analysis an unstained sample was run and used to exclude cell auto-fluorescence. Furthermore, for each analysis a minimum of 1000 events in the monocyte gate was used to acquire the FACS data.

2.7.2.3. Flowjo off line analysis

The commercial software FlowJo (Tree star, Ashland, Oregon) has been widely used in gating, visualization, and analysis of data from flow cytometry experiments. FlowJo was used to isolate different cell subsets and assess the level of median florescence of each population according to a specific marker**.** First finding regions of cells specific forward and side scatter such as lymphocytes and monocytes and then analysing these subsets under additional projections of fluorescence (Figure 15).

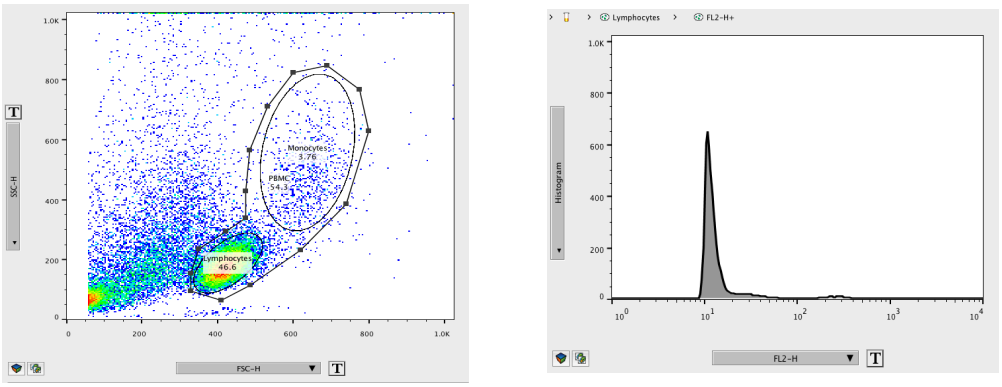


Figure 15 FlowJO off line analysis

2.8. Multiplex Analysis

Multi-Array® Technology (Meso Scale Discovery, MD USA) is a multiplex immunoassay system that enables the measurement of biomarkers using electrochemiluminescent detection. In an MSD® assay, specific capture antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system

uses patented SULFO-TAG™ labels, which emit light upon electrochemical stimulation initiated at the electrode surfaces of the Multi-Spot® plates. The electrical stimulation is decoupled from the output signal, which is light, to generate assays with minimal background. MSD labels, which are stable and non-radioactive, can be conveniently conjugated to biological molecules. Additionally, only labels near the electrode surface are detected, enabling non-washed assays. The MSD assays only require minimal sample volume as compared to a traditional ELISA (which is limited by its inability to measure more than one analyte). With an MSD assay, up to 10 different biomarkers can be analysed simultaneously using as little as 10-25 µl of sample. These assays have high sensitivity, up to five logs of linear dynamic range, and excellent performance in complex biological matrices. This allows the measurement of native levels of biomarkers in normal and diseased samples without multiple dilutions. In order to investigate levels of vascular and pro-inflammatory biomarkers, we used two kits; the human pro-inflammatory 7-plex ultra-sensitive Kit the human vascular injury panel 1 Kit.

2.8.1. Human Pro-inflammatory 7–plex Ultra-sensitive Kit

The Human Pro-inflammatory 7-Plex Ultrasensitive Kit (cat no.K15008C-1) was used. This kit measures IFN-γ, IL-1β, IL-10, IL-12p70, IL-6, IL-8, and TNF-α and the manufacturer's range of detection for each analyte is 0.61-10,000pg/ml. (Figure 16).

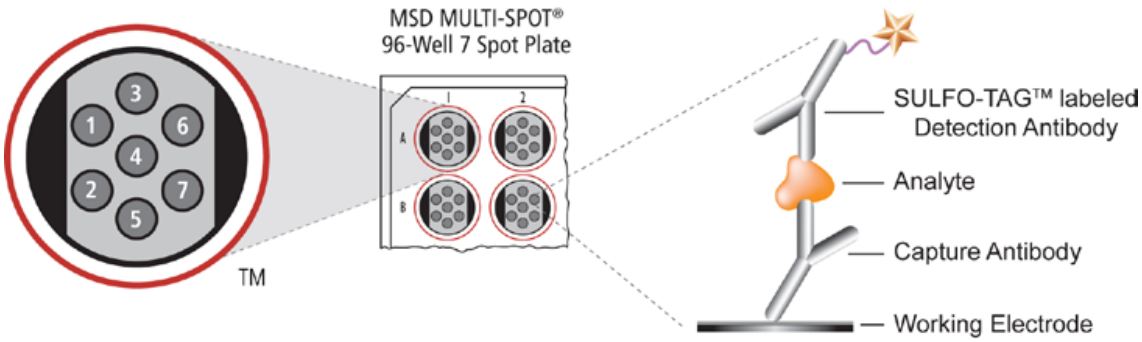


Figure 16 *MSD Human pro-inflammatory 7-plex*

In this project, Meso Scale analysis were done on EDTA samples collected during the study visits and stored in -80°C freezers. The assay was done using the human inflammatory 7-plex assay (Meso Scale Discovery) in accordance to manufacturer's instructions.

2.8.1.1. Assay procedure:

The MSD plates were coated with diluent 2 and incubated for 30 min with vigorous shaking (300–1000 rpm) at room temperature. Subsequently, 25 µl of each sample was added in duplicates to wells. This process was also followed for calibrators (cytokine detection controls), wherein 25 µl of each calibrator was plated out. Each plate had its own set of calibrators making detection more specific and accurate. The plates were then sealed and incubated for 2 hours with vigorous shaking (300–1000 rpm) at room temperature. After the incubation, the plates were washed with PBS-tween and detection antibody was added and incubated for 2 hours with vigorous shaking (300–1000 rpm) at room temperature. The plates were then washed again with PBS-tween. Subsequently, the read buffer was added to the wells and the plates were read by the MSD SECTOR Imager (MSD analyser). The analyser was adjusted to produce final results after considering the dilution factor.

The standards were reconstituted in the assay diluent provided. Assay diluent (25 µl) was added to all wells and the plate sealed and incubated for 30 s at room temperature on an orbital shaker (600 rpm). Samples, standards and controls were added at 25 µl per well. The plate was sealed and incubated for 2 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the wells were washed three times using 200 µl PBS+0.05%Tween 20, soaking for 30 s and then discarding. Detection antibody was added at 25 µl per well, and the plate sealed and incubated for 1 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the platewas washed three times as before. 150 µl of the MSD Read Buffer was added to each well and the MSD plates were measured on the MSD Sector Imager 2400 plate reader. The raw data was measured as electrochemiluminescence signal (light) detected by photodetectors and analysed using the Discovery Workbench 3.0 software (MSD). A 4-parameter logistic fit curve was generated for each analyte using the standards and the concentration of each sample calculated.

2.8.2. Human Vascular Injury I Kit

The Human Vascular Injury I 4-plex ELISA detects soluble intercellular adhesion molecule-3 (sICAM-3), E-selectin, P-selectin and thrombomodulin (TM) (Figure 17) in a sandwich immunoassay format (Meso Scale Discovery, MD USA). This immunoassay was conducted in the same manner as the Human Pro-Inflammatory Multi-Spot® plate as already described above.

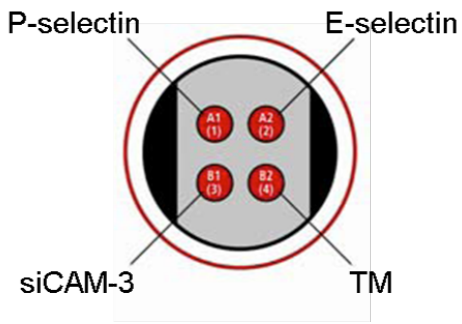


Figure 17 *MSD Vascular Injury I 4-plex*

2.9. Heme Oxygenase-1 (HO1) ELISA

HO-1 concentration was determined using a HO-1 (human) enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs Inc. Ann Arbor, MI). One hundred µl of plasma was incubated in the wells of the immunoassay plate, which was pre-coated with anti-HO-1, at room temperature for 30 min. After excess protein was decanted and washed with wash buffer, 100µ l of anti-human HO-1 was added to each well and the plate was incubated at room temperature for a further 1 h. After washing, horse radish peroxidase conjugated anti-rabbit IgG secondary antibody (100µ l) was added and the plate was incubated at RT for 30 min. After washing, the assay was developed by adding of 100µ l of tetramethylbenzidine substrate. The intensity of the color was measured in a microplate reader at 450nm.

2.10. LPS assay

The use of Limulus Amebocyte Lysate (LAL) for the detection of endotoxin evolved from the observation by Bang that a Gram-negative infection of Limulus polyphemus, the horseshoe crab, resulted in fatal intra-vascular coagulation, even if the bacteria were killed[573]. Levin and Bang later demonstrated that this clotting was the result of a reaction between endotoxin and a clottable protein in the circulating blood cells

(amoebocytes) of Limulus[574,575]. Following the development of a suitable anticoagulant for Limulus blood, they prepared a lysate from washed amebocytes which was an extremely sensitive indicator of the presence of endotoxin. This led to the production and commercialisation of the LAL assay for endotoxin detection in biological samples. According with this assay, Gram-negative bacterial endotoxin present in the samples catalyses the activation of a proenzyme in the LAL. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyses the splitting of pNA from the synthetic colourless substrate Ac-lle-Glu-Ala-Arg-pNA, producing a yellow colour. This is measured photometrically at 405-410 nm, after the reaction is stopped with stop reagent. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1-1.0 EU/ml range. The concentration of endotoxin in a sample is calculated from the absorbance values of solutions containing known amounts of endotoxin standard.

2.10.1.1. Assay procedure:

The test was performed using 96 well plates which were pre-equilibrated at 37° C in a heating block adapter. Meanwhile, four dilutions of the lyophilized endotoxin at a standardized concentration provided with the kit were prepared for the determination of the standard curve. The lyophilized endotoxin was reconstituted by adding 1 ml of pre-warmed LAL Reagent Water provided with the kit. As the concentration of lyophilized endotoxin may vary from 15-40 EU depending on the lot number, the actual concentration of endotoxin after this step corresponded to the value stated on the certificate analysis. For example, if the value reported on the vial of lyophilized endotoxin was 20 EU, after reconstitution with 1 ml of LAL Reagent Water, the concentration of the endotoxin stock was 20 EU/ml. The first dilution containing 1.0

EU/ml was prepared in a suitable container by diluting 0.1 ml of the endotoxin stock solution with (κ-1)/10 ml of LAL Reagent Water, where 'κ' equals to the concentration of endotoxin in the stock solution. For example, if κ=20 EU/ml, then 0.1ml of κ was diluted in 1.9ml of LAL Reagent Water, (20-1)/10ml, to obtain the first dilution containing 1.0 EU/ml of endotoxin. Following, 0.5 ml of this 1 EU/ml solution were transferred in a new container and 0.5 ml of LAL Reagent Water was added to obtain the second endotoxin dilution (0.5 EU/ml). Similarly, 0.5 ml of the 1 EU/ml solution was transferred in a vial containing 1.5 ml of LAL Reagent Water to obtain the third endotoxin dilution (0.25 EU/ml). Finally, 0.1 ml of the 1 EU/ml solution was transferred in a vial containing 0.9 ml of LAL Reagent Water to obtain the last dilution of endotoxin (0.1 EU/ml). Once all dilutions were prepared, 50 µl of the target sample or of the dilution series were added in duplicate into the appropriate microplate well. Each series contained a blank well control, where the samples or dilutions were substituted with 50 µl of LAL Reagent Water. At time T=0, 50µl of LAL were added to the first column of microplate wells using a multi-channel pipettor and reagent reservoir. The sequence of column as the LAL is added was taken into account in order to be consistent in the order of reagent addition from row to row, and in the rate of pipetting. Once the LAL was dispensed into all microplate wells containing samples (or standards), the microplate was briefly removed from the heating block adapter and repeatedly tapped on its the sides to facilitate mixing. The plate was returned to the heating block adapter and covered. Following 10 minutes, 100 µl of substrate solution (prewarmed to 37°C ± 1°C) were added. The substrate solution was pipetted following the same order of the LAL and trying to maintain a consistent pipetting rate. Once completed the addition of the substrate solution, the microplate was briefly removed from the heating block adapter and repeatedly tapped to facilitate mixing. The plate

was then returned to the heating block adapter and covered. The addition of the substrate caused a progressive change in the colour of the solution, which became yellow. Following 16 minutes, 50µl of stop reagent was added maintaining the same pipetting order as in of LAL and substrate addition. The plate was again removed from the heating block and tapped to allow proper mixing. The absorbance of each microplate well was read at 405-410nm, using distilled water to adjust the photometer to zero absorbance. Serum inhibition of LPS detection with the LAL tests is possible, particularly when using the colorimetric assay. This inhibition results in a lower, final Δ absorbance, indicating lower levels of endotoxin than what may actually be present in the test sample. Therefore, the lack of product inhibition should always be tested. In this thesis, the possible inhibition of the LAL reaction was determined for each sample using the method reported in the kit instruction. To verify the lack of product inhibition, an arbitrary dilution (1:5) of test sample was spiked with a known amount of endotoxin (0.4 EU/ml). To prepare a 0.4 EU/ml endotoxin solution in the diluted sample, the 1.0 EU/ml solution was diluted 1:2.5 using the diluted sample as the diluent. The spiked solution was assayed along with the unspiked samples and their respective endotoxin concentrations were determined. If the difference between these two calculated endotoxin values was not equal to the known concentration of the spike ± 25%, this suggested the presence of inhibitory factors to the LAL reaction in the sample and the sample was further diluted before the LPS assay. Using this method, the coefficient of variation obtained from a randomly selected set of 30 samples of study 2 which were run in two different days was 3.27%

2.11. Remote Ischemic Pre-Conditioning (RIPC)

The participants of study 4 were randomised to either RIPC group or either the control group consisting in a sham RIPC. RIPC was induced by transient limb ischemia achieved by inflating a standard 9 inch blood pressure cuff to 200 mmHg on the upper arm for five minutes, for three cycles each separated by a reperfusion period of five minutes, during which time the cuff was kept deflated. A similar protocol has been described by Kharbanda et al. to induce remote endothelial preconditioning in the contra-lateral limb in normal human volunteers [576]. The ischemia was induced on the right arm brachial artery and commenced just before the periodontal treatment since it has been shown that the endothelial protection conferred by RIPC disappears after 4 hours of preconditioning stimulus[577].

2.12. Statistics (General Methods)

All data are expressed as mean±SD unless otherwise stated. A number of parametric and non-parametric tests were used in this thesis. Parametric tests were used whenever possible (i.e when the data were normally distributed, or could be easily transformed to normality). If the data could not be easily transformed, non-parametric tests were performed. Normality of the data was assessed by plotting histograms and applying the Kolmogorov-Smirnov test of normality. Stata version 11 and SPSS version 22 analysis packages were used for the analysis of the data. Parametric tests used were independent t-test, paired t-test and two-way analysis of variance (TWO-WAY ANOVA) with treatment group and time as two main factors and with Bonferroni post hoc corrections. In all studies significance was defined as α-value of less than 5%, indicating that the chance of the null hypothesis still being true even though the difference is greater than the critical value was less than 5%

2.13. Ethics

All studies presented in this thesis were approved by local research Ethics Committee.

All study participants gave informed consent.

3. STUDY 1: ASSOCIATION BETWEEN PERIODONTAL DISEASE AND ITS TREATMENT, FLOW-MEDIATED DILATATION AND CAROTID INTIMA-MEDIA THICKNESS: A SYSTEMATIC REVIEW AND META-ANALYSIS

3.1. Introduction

Preliminary electronic searches of Medline, EMBASE and Cochrane Library identified several studies pertaining to periodontal disease and of markers of vascular health, such as c-IMT and FMD, consisting of observational and intervention studies[12,393,476,505,512,513]. No systematic reviews and meta-analysis were located. Research synthesis provides the background and context for new studies and new studies feed information back into the pool of data used in research synthesis. Therefore, a systematic review was an important project in itself and to guide further research.

3.1.1. Question Formulation

After consideration of population, exposures and outcomes potentially meaningful to the review, the review question was set as follows:

"What is the association between Periodontitis and vascular function as assessed by c-IMT and FMD and what is the effect of periodontal disease treatment on these parameters?"

3.1.2. Objectives and Null Hypotheses

Objectives and null hypotheses investigated are listed in Table 13. Further elaboration of the rationale for these is found in the following section.

Table 13 Objectives and null hypothesis

Objective category	Null Hypothesis
Primary	- to test the null hypothesis of no association periodontitis and the c-IMT in adults - to test the null hypothesis of no association periodontitis and the FMD in adults - to test the null hypothesis of no association between c-IMT and periodontal therapy in adults - to test the null hypothesis of no association between FMD and periodontal therapy in adults
Secondary	- to report factors considered to be confounding factors

3.2. Methods

Design aspects of the review were outlined in a detailed protocol addressing the aims, objectives, inclusion/exclusion criteria, search and data extraction strategy, risk of bias assessment, and synthesis of extracted evidence.

3.2.1. Scope

The purpose of the review was: i) to investigate, by means of a meta-analysis, whether current data support an association between PD, increased c-IMT and impaired FMD, ii) whether periodontal treatment has any effect on these variables and iii) summarize the existing evidence of an association between PD (the exposure) and vascular alterations (the outcomes). PD was defined to include any measure of periodontal

disease according to clinical, radiographic and microbiological assessment. This includes measures of Pocket Probing depth (PPD), clinical attachment level (CAL), bleeding on probing (BOP), Plaque index (PI), Gingival index (GI), X-ray and microbiological assay for periodontal pathogens as reported. Surrogate markers of CVD eligible for inclusion were: B-ultrasound measurements of c-IMT, expressed as Mean (SD) and FMD as Mean (SD) of the percentage or absolute value of dilation. We included individuals aged ≥ 17.

3.2.2. Study inclusion/exclusion criteria

In order to evaluate potential associations between PD, c-IMT and FMD, inclusion criteria were set to be broad and inclusive. Both observational and experimental designs were deemed eligible including case-control, cross-sectional, cohort studies, population surveys, pilot studies, controlled trials and randomized controlled trials. Comments, letters, editorials, case studies and series, news items, abstracts not followed by publication and consumer health material were excluded. Only studies performed in humans and containing measures of either c-IMT (acquired by B-mode ultrasound and expressed as Mean ± SD) or FMD (performed using standard protocols of ultrasound imaging and reported as % or mm of dilatation of the brachial artery compared to the baseline diameter) were included. Other studies not eligible for inclusion in this review were: those performed in animals, duplicated reports (i.e. studies originating from the same study samples by the same investigators but published in different journals), studies providing associations between different markers of CVD or particular pathogens involved in PD and CVD, studies defining endothelial function with different methodology than FMD as well as those providing the c-IMT in a different artery of the carotid or providing data with regards to other morphologic characteristics.

3.2.3. Search and screening

Review articles as well as relevant narrative or systematic reviews represented the preliminary search to assist in formulation of the research strategy and confirm no existing systematic reviews. The search strategy was developed using medical subject headings (MeSH terms) as well as free text terms and was customized as appropriate before application to each database. An example of the search strategy implemented is shown in Table 14.

Table 14 EMBASE (OVID) Search strategy example
1. exp periodontitis/ or exp periodontal disease/ or periodont*.mp. or exp periodontium/
2. periodontal attachment loss.mp.
3. oral hygiene.mp. or exp mouth hygiene/
4. 1 or 2 or 3
5. exp cardiovascular disease/
6. exp ATHEROSCLEROSIS/ or exp CORONARY ARTERY ATHEROSCLEROSIS/
7. exp carotid artery disease/
8. 5 or 6 or 7
9. exp brachial artery/
10. exp ENDOTHELIUM INJURY/ or exp ENDOTHELIUM CELL/ or exp ARTERY ENDOTHELIUM/ or exp ENDOTHELIUM/ or exp ENDOTHELIUM LESION/ or exp VASCULAR ENDOTHELIUM/
11. exp endothelial dysfunction/ or exp vasodilatation/ or endothelial function.mp. or exp acetylcholine/
12. intima-media thickness.mp. or exp arterial wall thickness/
13. exp artery blood flow/
14. tunica intima.mp. or exp intima/
15. tunica media.mp. or exp tunica media/
16. 9 or 10 or 11 or 12 or 13 or 14 or 15
17. 8 and 16
18. 4 and 17
19. 18 not animal.mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer]

In addition, bibliographic references were hand searched through the reference list of the identified papers and previous reviews. Online hand searching of issues within the past 10 years of some periodontal, cardiovascular and imaging journals was also performed (Table 15).

Table 15 Periodontal and Mediacl Jopurnals Hand-searching	
Periodontal Journals	**International Journal of Periodontics & Restorative Dentistry**
	Journal of Clinical Periodontology
	Journal of Periodontal Research
	Journal of Periodontology
	Periodontology 2000
Cardiovascular	**Circulation**
	European Heart Journal
	Cardiovascular Research
	Circulation Research
	Cardiology
	Arteriosclerosis, Thrombosis, and Vascular Biology
	American Journal of Cardiology
	Stroke
	Atherosclerosis
Ultrasound Imaging	**Cardiovascular ultrasound**
	Journal of cardiovascular ultrasound
	Journal of clinical ultrasound
	Ultrasound in medicine & biology
	Seminars in ultrasound, CT, and MRi
	Journal of ultrasound

Two investigators screened independently titles and abstracts. Full text articles were assessed after considering an abstract potentially suitable. The following databases were reviewed:

- The Cochrane Oral Health Group's Trials Register, (whole database to current date)
- The Cochrane Central Register of Controlled Trials (CENTRAL, whole database at current issue)
- MEDLINE via OVID, (1946 to present)
- EMBASE via OVID, (1980 to present)
- SCI-EXPANDED via Web of Science, (1899 to present)
- LILACS via VHL, (1982 to present)
- System for Information on Grey Literature in Europe (Open SIGLE, 1980-2005)

This systematic review was written according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines[578].

3.2.4. Bias protection assessment

Bias-protection assessment of included studies was undertaken independently and in duplicate by two reviewers. Cohort and case–control studies were assessed for quality using the validated Newcastle-Ottawa Quality Assessment Scale as recommended by the Cochrane Collaboration Guidelines for the assessment of non-randomized studies. This tool awards stars (*) in three categories for each study based on incorporation of design elements associated with minimising bias. A validated assessment tool to assess cross-sectional studies was not located. The quality of cross-sectional studies were assessed using questions from the Newcastle-Ottawa Quality Assessment Scale for cohort study design that were deemed applicable to the cross-sectional study design[579].

3.2.5. Data extraction

Data abstraction forms were developed and piloted on three studies by both investigators, then revised to increase the accuracy of the collection process. All included studies were analyzed independently and in duplicate. Completed forms were compared to validate accuracy of the data abstraction. Disagreements were resolved through discussion with a third person.

3.2.6. Data synthesis

3.2.6.1. Descriptive methods

Data were then analyzed using descriptive and quantitative methods. When descriptive methods were used, all publication data were summarized in evidence tables in order to analyze any difference in study characteristic and to quantify the body of evidence. The following data were collected from each research article: name of the first author, year of publication, sample size, demographic characteristics, covariates included for the adjustment, assessment of dependent and independent variables, study outcome. Studies were grouped according to design in the evidence tables to facilitate assessment of suitability for inclusion in the planned meta-analysis. If the publications did not contain all information necessary for meta-analysis, authors were contacted to retrieve missing information.

3.2.6.2. Quantitative methods

Statistical analyses were performed using Stata (Version 10, StataCorp). Mean differences in both c-IMT and FMD were used to perform the following meta-analyses; c-IMT mean value in individuals with PD compared to controls in case-control and cross-sectional studies and FMD mean value in individuals with or without PD as well as before and after periodontal treatment. If case data were not available, the missing

information was obtained directly from the authors (see Acknowledgements). The pooled estimates of the mean differences were calculated using random effects models to take into account a potential inter-study heterogeneity and then adopt a more conservative approach. The pooled effect was considered significant if p was < 0.05. Forest plots for each meta-analysis present the raw data (i.e., means, SDs, and sample sizes), point estimates (displayed as blocks), and CIs (displayed as lines) for the chosen effect, heterogeneity statistic (I-squared), total number of participants per group, overall average effect, and the weight given to each study. Small study bias was examined using funnel plot and Egger's test. Sensitivity analyses were performed to understand the influence of individual studies, by omitting one study each time in a series of meta-analyses to identify influential studies.

3.2.7. Heterogeneity

Heterogeneity was assessed by using the χ2-based Q-statistic method, considered significant if $P < 0.05$[580]. Heterogeneity was also quantified with the I-squared statistic, a value that indicates what proportion of the total variation across studies is beyond chance, where 0% indicates no observed heterogeneity and larger values show increasing heterogeneity[581].

3.3. Results

2066 hits were obtained from electronic searches and 2 papers were located via on line and hand searching of mentioned journals of interest. Following removal of 59 duplicates, 2009 potentially suitable titles and abstracts were screened in duplicate. After excluding 1906 non-relevant citations, 101 publications were selected and retrieved for full-text review (Figure 18).

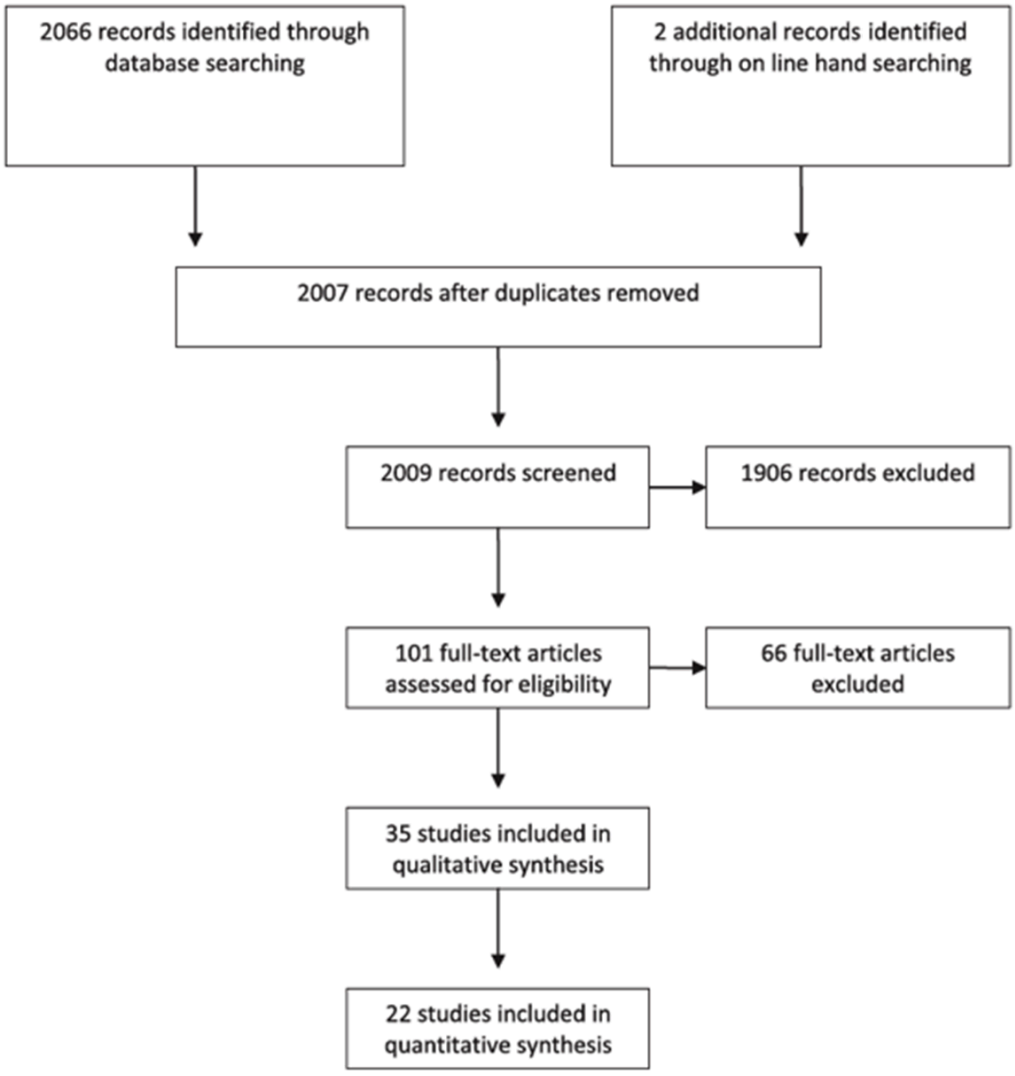


Figure 18 *Flow chart of the study selection procedure*

Among these full text articles and abstracts, 66 were excluded; 30 were reviews or editorials and 36 did not report outcomes relevant to the review. This resulted in a total of 35 publications eligible to be included in the systematic review after the screening procedure. Of these, 22 papers were included in the meta-analyses. 6 publications did not contain the full information necessary for the meta-analyses[476,512,582-585], however it was possible to obtain the required data from the authors. All 35 studies reported evidence on an association between investigated CVD markers and PD.

3.3.1. Descriptive results

The full body of data obtained from the included studies was tabulated according to the chronological date of publication and sorted according to the outcome measure and study design (Tables 16 to 19)

3.3.1.1. PD and c-IMT

26 publications included measures of c-IMT of which 25 were observational studies (Table 16) and 1 intervention trial (Table 17). There was a considerable variation in terms of definition of PD, observed population, study sample size, age range, carotid segments studied and definition of c-IMT among the studies. 16 publications were included for further analysis as 10 did not provide estimates that were comparable to the other studies. The definition of the carotid segments was the major source of heterogeneity. 1 uncontrolled longitudinal study evaluated the impact of periodontal treatment on c-IMT in individuals with mild or moderate PD. After a follow up of 6 and 12 months this study showed a reduction of c-IMT compared to the BL measurements[399].

3.3.1.2. PD and FMD

The relationship between PD and FMD was investigated in 10 articles of which 3 were observational (Table 18), and 7 intervention studies (Table 19). 2 randomized controlled trials were not suitable for the meta-analysis as endothelial function was assessed measuring the response of the brachial artery blood flow to acetylcholine instead of FMD[375,413] and one intervention pilot trial had no control group[507].

3.3.2. Bias assessment protection

According to the Newcastle-Ottawa Quality Assessment Scale for the relevant study design, the majority of the studies met the criteria to be categorized as low or medium

risk of bias within the respective study design (Figure 19). There was a discrepancy in the periodontal diagnosis, definition of controls and not a uniform adjustment for potential confounders. In case-control studies, individuals included in control groups were recruited from hospital rather than from the community, reducing the quality in the selection. All cross-sectional studies were considered comparable in quality and the same for controlled trials.

	Selection	Comparability	Exposure	Overall
Case-control c-IMT				
Leivadaros 2005	***	**	***	********(8/9)
Soder 2005	****		***	*******(7/9)
Soder 2007	****		***	*******(7/9)
Cairo 2008	***	**	***	********(8/9)
Lopez J 2012	***		***	*****(6/9)
Zahnd 2012	***	**	***	******(6/9)
Puhar 2012	***		***	******(6/9)
Cross-sectional c-IMT				
Beck 2001	***	**	***	********(8/9)
Franeck 2006	***		***	******(6/9)
Li X 2009	***		***	******(6/9)
Ylostalo 2010	***	*	***	*******(7/9)
LI P 2011	***		***	******(6/9)
Vieira 2011	***	*	***	*******(7/9)
Franek 2012	***	**	***	********(8/9)
Pinho 2013	***	**		
Hayashida 2013	***		***	********(8/9)
Case-control-FMD				
Amar 2003	**	**	***	********(8/9)
Ruiz 2013	***		***	******(6/9)
Cross-sectional-FMD				
Li 2011	***		***	******(6/9)

Figure 19 *Quality assessment observational studies (Newcastle-Ottawa Scale).*

Table 16 Characteristics of observational studies included in the qualitative analysis, c-IMT outcomes

First Author (Year/Country) study deign	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Leivadarios E. [476] **(2005/Netherlands) Case-control Included in the quantitative results**	N = 63 (Control = 14; Mild/Moderate periodontitis = 34; Severe periodontitis = 15) University/Hospital setting Age range = not reported Case definition = Severe periodontitis as ≥7 teeth with ≥50% bone loss Control definition = 1 or no missing teeth per quadrant (third molars excluded). a radiographic distance ≤3 mm between the cemento-enamel junction (CEJ) and the alveolar bone crest on all teeth.	Age, sex, race BMI, cholesterol, syst/diastolic BP	Intima-media thickness of the carotid artery (c-IMT) Bilateral, common (CCA) bifurcation (BCA), internal (ICA) and overall IMT. The far (posterior) wall of the vessel along a 1cm section. Measurements were the mean of the six segments.	Radiographic examination with Schei ruler technique	Unadjusted means (mm) for c-IMT: - CCA Control: 0.64 Mild/Moderate: 0.68 Severe: 0.69 p = 0.749 - ICA Control: 0.58 Mild/moderate: 0.55 Severe: 0.81 p = 0.023 - Overall IMT Control: 0.65 Mild/Moderate: 0.64 Severe: 0.76 p = 0.153 Adjusted means (mm) for IMT (95% CI) ANCOVA model - CCA Control: 0.64 (0.53-0.74) Mild/moderate: 0.64 (0.50-0.73) Severe: 0.64 (0.54-0.75) P= 0.990 - BCA Control: 0.72 (0.59-0-85) Mild/moderate: 0.66 (0.56-0.78) Severe: 0.71 (059-0.83) P= 0.634 - ICA Control: 0.61 (0.43-0.78) Mild/Moderate: 0.52 (0.37-0.66) Severe: 0.76 (0.59-0.93) P= 0.040 - Overall IMT Control: 0.65 (0.55-0.76) Mild/Moderate: 0.61 (0.53-0.69) Severe: 0.70 (0.60-0.81) P= 0.233

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Söder P-Öss[586] (2005/Sweden) case-control Included in the quantitative results	N = 113 (Test = 82 [78 analysed]; Control = 31) Population based sample Age range = 30 – 40 Case definition = Periodontitis defined as: at least 1 site with PD ≥ 5 mm Control definition = Periodontally healthy	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) bilateral, common carotid artery (CCA) Far wall, 0.5-1 cm proximal to the carotid bulb.	PD, PI, GI,CAL, X-ray	c-IMT mean ± SD (mm) - Common carotid artery (right side) Cases 0.66 ± 0.12 Controls 0.58 ± 0.09 p <0.01 - Common carotid artery (left side) Cases 0.68 ± 0.12 Controls 0.58 ± 0.08 p <0.001
Söder B.[587] (2007/Sweden) case-control Included in the quantitative results	N = 67 (Test= 46 [43 analysed]; Control = 21 only women) Uni/Hospital setting Age range = 30-40 Case definition = No definition of periodontitis Control definition = Periodontally healthy	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) bilateral, common carotid (CCA) Far wall, 0.5-1 cm proximal to the carotid bulb.	PD, PI, GI, CAL, X-ray	c-IMT mean ± SD (mm) -Common carotid artery (right side) Cases 0.65 ± 0.11 Controls 0.60 ± 0.09 p ≤ 0.05 - Common carotid artery (left side) Cases 0.67 ± 0.11 Controls 0.58 ± 0.08 p < 0.001

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Cairo F.[513] (2008/Italy) Case-control Included in the quantitative results	N=90 (Control= 45; Test = 45) Uni/Hospital setting Age range = 18-40 Case definition = Severe periodontitis as: At least 30% sites with CAL and alveolar bone loss exceeding 1/3 of the root in at least 30% of the entire dentition Control definition = Otherwise healthy subjects with CAL ≤ 3mm at each site	Age, sex, smoking habits	Intima-media thickness of the carotid artery (c-IMT) Bilateral, common carotid (CCA) Far wall real-time measurement of carotid IMT represented the mean of 10 measures on each side	PD, FMPS, FMBS, CAL	c-IMT mean ± SD(mm) ; 95%CI Cases 0.82 ± 0.13; [0.61-1.16] Controls 0.72 ± 0.07; [0.59-0.94] p < 0.0001
Cairo F.[588] (2009/Italy) Case-control	N = 90 (Control = 45; Test = 45) Uni/Hospital setting Age range = 18-40 Case definition = Severe periodontitis as: At least 30% sites with CAL and alveolar bone loss exceeding 1/3 of the root in at least 30% of the entire dent. Control definition = Otherwise healthy subjects with CAL ≤ 3mm at each site	Age, sex, smoking habits	Intima-media thickness of the carotid artery (c-IMT) Bilateral, common carotid (CCA) Far wall real-time measurement of carotid IMT represented the mean of 10 measures on each side	PD, FMPS, FMBS, CAL	c-IMT mean ± SD (mm) Cases 0.82 ± 0.13 Controls 0.72 ± 0.07 p < 0.0001

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Söder P-Ö[589](26) **(2009/Sweden)** **Case-control**	N= 111 (Test = 80; Control= 31) Population based sample Age range = not reported Case definition = no definition of periodontitis Control definition = Periodontally healthy	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) bilateral, common carotid (CCA) Far wall, 0.5-1 cm proximal to the carotid bulb.	PD, CAL, BOP, GI, PI	c-IMT mean ± SD (mm) -Common carotid artery (right side) Cases 0.66 ± 0.12 Controls 0.58 ± 0.09 p < 0.01 - Common carotid artery (left side) Cases 0.68 ± 0.12 Controls 0.58 ± 0.08 p < 0.001
Lòpez N.G.[590] **(2011/Chile)** **Case-control**	N = 60 (Test = 55; Control= 53) Population with various conditions Uni/Hospital setting Age range= not specified Case definition= Periodontitis as: At least 6 teeth with PD ≥ 4 mm and AL ≥ 3 mm Control definition = Moderate to severe periodontitis treated at least since 10 years, regular maintenance session every 6 months, pre-trt x-rays showing at least 30% of bone loss.	Age, gender, smoking, education, diabetes, physical activity, vegetables intake, BMI, TC, LDL, HDL, alcohol, statin, aspirine, blood pressure	Intima-media thickness of the carotid artery (c-IMT) Bilateral, bifurcation(BCA,1 cm proximal to the flow divider), internal(ICA, 1cm distal to the flow divider, common (CCA, 1cm proximal to the dilatation of the carotid bulb), the carotid bifurcation and the internal carotid artery) Measurements were the mean of the six segments.	PD, BOP, CAL, FMPS	c-IMT mean ± SD (mm) Cases 0.775 ± 0.268 Controls 0.683 ± 0.131 p = 0.027

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Lòpez-Jornet P.[591] **(2012/Spain) Case-control Included in the quantitative results**	N = 60 (Test = 30; Control= 30) Otherwise healthy population Uni/Hospital setting Age range= 35-70 Case definition= Chronic periodntitis as: ≥16 teeth and ≥10 sites with PD of >5 mm. CAL >3mm: 1% to 32%= mild 33% to 66% = moderate 67% to 100% = severe Control definition = Otherwise healthy without PD	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) right common carotid artery (CCA) the far (posterior) wall of the vessel along a 1cm section proximal to the bifurcation. 3 measurements taken, maximum IMT measurement was recorded.	PD, CAL, BOP,	c-IMT mean ± SD (mm) Cases 0.77 ± 0.17 Controls 0.81 ± 0.27 p = 0.538

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Zahnd G.[592] (2012/Australia) Case-control Included in the quantitative results	N = 152 (Test = 125; Control= 27) Subset of PerioCardio study Age range= not reported Case definition= Moderate periodontitis defined as: presence of either two sites between adjacent teeth with ≥4 mm attachment loss or at least two such sites with ≥5 mm pockets. Severe periodontitis: at least two sites between adjacent teeth with ≥6 mm attachment loss and at least one pocket ≥5 mm. Control definition = healthy non-smokers volunteers,	Age, sex	Intima-media thickness of the carotid artery (c-IMT) Case= Bilateral, common carotid (CCA), far wall, within 10 mm proximal to the carotid bulb. Controls = Left common carotid only data for the left carotid artery were used for comparative analysis.	PD, CAL	c-IMT mean ± SD (mm) Cases 0.64 ± 0.17 Controls 0.57 ± 0.09 p = 0.007
Puhar I.[585] (15) (2012/Croatia) Case-control Included in the quantitative results	N = 128 (Test = 67; Control= 61) Uni/Hospital setting Age range= not reported Case definition= CAL ≥3 mm at ≥30% of sites was used to define generalized forms of periodontitis. Control definition = Periodontally healthy	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) bilateral,common carotid artery (CCA)	PD, CAL, BOP, PI, Rec	c-IMT mean ± SD (mm) Cases 0.7 ± 0.21 Controls 0.59 ± 0.21

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Beck J.D.[512] (2001/USA) **Cross-sectional** **Included in the quantitative results**	N= 6017 Subset of Atherosclerosis Risk in Communities (ARIC) In 1996-1998 Conducted at ARIC visit 4 Age range=52-75 Case definition: None/Mild (<10% sites with AL≥3mm) Moderate (10% to <30% sites with AL≥3mm) Severe (≥30% sites with AL≥3mm) Extent of AL also divided into quintiles (<3.7%, 3.7%to <8.7%, 8.7% to<16.7% 16.7% to 30.9%, ≥30.9%)	Age, sex, diabetes, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, hypertension, smoking, waist/hip ratio, education, and race/center	Intima-media thickness of the carotid artery (c-IMT) Bilateral, bifurcation (BCA), internal (ICA), common (CCA). The far (posterior) wall of the vessel along a 1cm section. Measurements were the mean of the six segments.	CAL, PD, REC	c-IMT mean ± SD (mm) Severe periodontitis 0.82 ± 0.24 Moderate periodontitis 0.77 ± 0.22 None / Mild 0.74 ± 0.19 p = 0.0001 IMT ≥1 mm OR, 95% CI - Unadjusted None / mild 1.00 (referent) Moderate 1.40, 1.17-1.67 Severe 2.09, 1.73-2.53 - Adjusted None / mild 1.00 (referent) Moderate 1.10, 0.89 – 1.35 Severe 1.31, 1.03 – 1.66

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Desvarieux M.[593] (2004/Germany) Cross-sectional	N= 1710 Subsample of Study of Health in Pomerania (SHIP) Age range= 45-75 Case definition: Subjects categorized in tertiles according to: -TOOTH LOSS 0 to 8, 9 to 15, 16 to 31 -% of SITES WITH AL≥4 mm 0-20%, 21-57%, ≥57%	Age, region, smoking, diabetes, systolic blood pressure, high blood pressure, LDL-C, HDL-C, tryglicerides, education, BMI	Intima-media thickness of the carotid artery (c-IMT) Bilateral, common carotid (CCA), far wall the mean far-wall IMT was calculated by averaging the 10 consecutive measurement points.	PD, CAL, REC using the half-mouth method on the right or left-side in alternate subjects.	Gender difference in c-IMT means ± SE (mm) with a cut-off of AL≥5 mm: Males Tertile I 0.79 ± 0.1 Tertile II 0.80 ± 0.001 Tertile III 0.82 ± 0.009 P= 0.05 (highest/lowest tertile) Females Tertile I 0.74 ± 0.009 Tertile II 0.74 ± 0.009 Tertile III 0.73 ± 0.009
Desvarieux M.[594] (34) (2005/USA) Cross-sectional	N= 657 Subsample from The Oral Infections and Vascular Disease Epidemiology Study (INVEST) Age range = not reported Case definition: Microbiological assessment - Cumulative Periodontal bacterial burden - Etiologic bacterial burden - Bacterial dominance	Age, BMI, sex race, education systolic BP, LDL and HDL cholesterol smoking, diabetes	Intima-media thickness of the carotid artery (c-IMT) common, bifurcation, internal. c-IMT was calculated as a composite measure (mean of the 12 sites) that combined the near and the far wall of the maximal common carotid artery IMT, the maximal bifurcation IMT, and the maximal internal carotid artery IMT bilaterally.	PD, BOP, PI	c-IMT mean ± SE (mm) across increasing tertiles of perio bacterial exposure definitions Cumulative burden P for linear trend 0.04 Tertile I 0.84 ± 0.008 Tertile II 0.86 ± 0.008 Tertile III 0.87 ± 0.008 Etiologic burden P for linear trend 0.03 Tertile I 0.84 ± 0.010 Tertile II 0.86 ± 0.008 Tertile III 0.87 ± 0.010 Etiologic dominance P for linear trend 0.002 Tertile I 0.84 ± 0.008 Tertile II 0.85 ± 0.007 Tertile III 0.88 ± 0.008

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Beck J.D.[595] **(2005/USA) Cross-sectional**	N= 4585 Subset of Atherosclerosis Risk in Communities (ARIC) Conducted at ARIC visit 4 In 1996-1998 Age range = 45-64 Case definition: IgG antibody levels to 17 selected periodontal organisms	Age, sex, race, diabetes, Hypertension, waist-to hip ratio, HDL, LDL, trygliceride, education	Intima-media thickness of the carotid artery (c-IMT) Bilateral, bifurcation, internal, common. The far (posterior) wall of the vessel along a 1cm section. Measurements were the mean of the six segments.	CAL, PD, REC	High IgG level and c-IMT ≥1 OR (95% CI) Ever smokers **Never smokers** N=2174 **N=2895** P. gingivalis 1.5 (1.2-2.1) 0.0017 - **1.4 (1.0-1.9) 0.0848** T. forsysthensis 1.0 (0.8-1.3) 0.9568 - **1.3 (0.9-1.8) 0.0936** T. denticola 1.3 (1.0-1.7) 0.0497 - **1.6 (1.2-2.3) 0.0055** P. intermedia 1.3 (1.0-1.6) 0.0973 - **1.8 (1.2-2.5) 0.0016** C. rectus 2.3 (1.7-3.0) <0.0001 - **2.2 (1.5-3.2) <0.0001** P. micros 1.8 (1.4-2.4) <0.0001 - **2.2 (1.6-3.1) <0.0001** P. nigrescens 1.2 (0.9-1.5) 0.3034 - **1.3 (0.9-1.8) 0.1673** F. nucleatum 1.9 (1.4-2.5)<0.0001 - **1.9 (1.3-2-7) 0.0004** S. noxia 1.8 (1.4-2.4) <0.0001 - **2.0 (1.4-2.8) 0.0001** A. actinmomycetemcomitans 1.6 (1.2-2.1) 0.0012 **1.6 (1.2-2.3) 0.0058** E. corrodens 1.9 (1.4-2.5) <0.0001 - **2.6 (1.8-3.8) <0.0001** C. ochracea 2.0 (1.5-2.6)<0.0001 - **1.8 (1.3-2.6) 0.0007** V. parvula 2.0 (1.5-2.7) <0.0001 - **2.4 (1.7-3-5) <0.0001** S. sanguis 1.4 (1.1-1-8) 0.0170 - **1.9 (1.4-2.7) 0.0002** S. intermedius 1.0 (0.8-1-3) 0.8478 - **2.0 (1.4-2.9) <0.0001** S. oralis 1.4 (1.0-1.8) 0.0295 - **2.1 (1.5-3.0) <0.0001** A. viscosis 1.4 (1.1-1.8) 0.0155 - **1.7 (1.2-2.4) 0.0019**

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Pussinen P.[596] (2005/Finland) Cross-sectional	N= 1023 (male only) Subset of Kuopio Ischemic Heart disease Risk Factor Study (KIHD) Age Range = 46-64 Case definition: Serum antibody levels to periodontal pathogens Aa IgA/IgG, Pg IgA/IgG Subjects categorized in tertiles	Age, smoking plasma fibrinogen diabetes, BMI med for HBP socioeconomic status, serum HDL/LDL-C	Intima-media thickness of the carotid artery (c-IMT) bilateral, common carotid (CCA) far wall, Mean IMT was computed as the mean of 100 measurements in the right CCA and another 100 measurements in the left CCA.	Antibody levels to periodontal pathogens	c-IMT mean ± SD (mm) Own teeth 0.8459 ± 0.009 Denture 0.8844 ± 0.009 P<0.01 -Aa IgA Tertile I 0,8609 ± 0.009 Tertile II 0,8861 ± 0.015 P<0.01 Tertile III 0,8733 ± 0.02 P<0.05 -Aa IgG Tertile I 0,8609 ± 0,011 Tertile II 0,8574 ± 0,01 Tertile III 0,8853 ± 0,012 -Pg IgA Tertile I 0,8363 ± 0,009 Tertile II 0,8711±0,011 P<0.05 Tertile III 0,8988±0,013 P<0.001 -Pg IgG Tertile I 0,8443 ± 0,01 Tertile II 0,884±0,013 P<0.01 Tertile III 0,8769±0,01 P<0.05 -Aa IgG + Pg IgG Tertile I 0,818 ± 0,014 Tertile II 0,8271 ± 0,013 Tertile III 0,8829 ± 0,018 P<0.01

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Franek E.[514] (2006/Poland) Cross-sectional Included in the quantitative results	N= 44 (Test = 17 Control = 27) Uni/Hospital setting Age range = not reported Case definition = no definition of advanced chronic periodontitis	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) bilateral, common carotid	PD, CAL, PI, BI	c-IMT mean ± SD (mm) Test 0.742 ± 0.028 Control 0.656 ± 0.019 p < 0.05
Demmer R.T.[597] (2008/Germany) Cross-sectional	N= 1745, age≥ 45 Subsample from the Study Of Health in Pomerania (SHIP) (N=3557) Age range = 45-79 (SHIP age range= 20-79) Case definition = for both PPD and AL candidate definitions were defined for extent of PD severity thresholds ranging from 3-10 mm as follow: 1 number of sites ≥ a given severity threshold 2 percentage of sites ≥ a given severity threshold 3 sum of mm ≥ a given severity threshold 4 mean of mm ≥ a given severity threshold	Age	Intima-media thickness of the carotid artery (c-IMT) Bilateral, common carotid, far wall	PD, CAL using the half-mouth method on the right or left-side in alternate subjects. BOP on the first molar, the canine and the central incisor (up to 24 sites /mouth), tooth loss	c-IMT mean ± SE (mm) of across quintiles of selected periodontal disease definition Men (n=879) p- for linear trend 0.005 Quintile I 0.80 ± 0.01 Quintile II 0.81 ± 0.01 Quintile III 0.80 ± 0.01 Quintile IV 0.82 ± 0.01 Quintile V 0.84 ± 0.01 Women (n=866) p-for linear trend 0.03 Quintile I 0.73 ± 0.01 Quintile II 0.72 ± 0.01 Quintile III 0.72 ± 0.01 Quintile IV 0.76 ± 0.01 Quintile V 0.77 ± 0.01

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Li X.[516] (2009/China) Cross-sectional Included in the quantitative results	N=86 (Test = 63; Control = 23) Sample from a health screening programme Age Range = 35-80 Case definition = Chronic periodontitis defined if subjects fulfilled any of the following criteria: - more than 6 sites with PD≥ 4 mm - over 25% sites with interproximal CAL≥ 5 mm - more than 8 missing teeth due to CP excluding the third molars	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) 3 measurements were made on the near and far wall of the left and right common carotid, bifurcation and internal carotid. The mean maximum IMT (mmIMT) was calculated by averaging the values of maximum IMT measured from 12 preselected segments in the carotid arteries.	PD, CAL, REC, BOP, Missing teeth	c-IMT mean ± SD (mm) - No or Mild PD 0.75 ± 0.15 - Moderate to severe PD 0.83 ± 0.15 p = 0.039
Ylöstalo P.[598] (2010/Finland) Cross-sectional Included in the quantitative results	N = 60 Subsample of a population-based Cohort of those inhabitants of Oulu born in 1935 Age Range = not reported Case Definition: subjects categorized in 3 groups: 1-PD≤3 mm 2-at least 1 tooth with PD≥4 and ≤5 mm 3-at least 1 tooth with PD≥6 mm	Sex, diabetic status	Intima-media thickness of the carotid artery (c-IMT)	CPITN	c-IMT mean ± SD (mm) in relation to PD: 1- 0.93 ± 0.27 2- 0.96 ± 0.15 3- 0.93 ± 0.26
Vieira C.L.Z.[517] (2011/Brazil) Cross-sectional Included in the quantitative results	N= 79 (Test = 33 Control = 46) Patients with hFH followed At the Lipid Clinic of the University of Sao Paulo Age range = 17-80 Case definition = severe periodontitis as: ≥3 sites not on the same tooth CAL≥ 7 mm ≥1 interproximal sites with PD≥ 5 mm	Age, smoking, HDL, BMI, BP	Intima-media thickness of the carotid artery (c-IMT) right common carotid	PD, REC, CAL	c-IMT mean ± SD (µm) Severe periodontal group: 710.91 ± 164.64 Non severe periodontal group: 633.12 ± 157.94 p = 0.03

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Li P.[515] (2011/China) Cross-sectional Included in the quantitative results	N = 59 (MS group = 26) (non-MS = 33) Uni/Hospital setting Age Range = 23-76 (MS group) 37-71 (non-MS) Case definition = periodontal disease as CAL >3mm: 1% to 32%= mild 33% to 66% = moderate 67% to 100% = severe	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) Bilateral common carotid	PD, CAL, BI, PI, missing teeth	c-IMT mean ± SD (mm) - Non MS group *Left side* No/Mild PD 0.76 ± 0.27 Moderate/severe 0.85 ± 0.24 *Right side* No/Mild PD 0.67 ± 0.18 Moderate/severe 0.89 ± 0.37 - MS group *Left side* No/Mild PD 0.84 ± 0.33 Moderate/severe 1.08 ± 0.49 *Right side* No/Mild PD 0.78 ± 0.30 Moderate/severe 0.92 ± 0.49

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Franek E.[582] (2012/Poland) Cross-sectional Included in the quantitative results	N = 121 (BGI-H 16, BGI-G 87, BGI-P2 18) Diabetic population Uni/Hospital setting Age range = not reported Case definition = Differences in biologic phenotype Periodontitis as: PD ≥ 4 mm and BOP extent scores < 10% as BGI-P1; deep lesion/low bleeding, with BOP extent scores 10–50% as BGI-P2; and deep lesion/moderate bleeding and with BOP extent scores > 50% as BGI-P3-deep lesion/severe bleeding.	age, sex, body mass index (BMI), HbA1c, LDL-cholesterol, CRP and periodontal status.	Intima-media thickness of the carotid artery (c-IMT) bilateral, common carotid. 3 recordings each left and right artery. The mean value was calculated for each side and for all six measurements.	BOP, PD	c-IMT mean ± SD (mm) BGI-P2: 0.804 ± 0.112 BGI-G: 0.772 ± 0.127 BGI-H: 0.691± 0.151 p < 0.01 BGI-P2 compared to BGI-H group

Table 16 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Pinho M.[584] **(2013/Portugal) Cross-sectional Included in the quantitative results**	N = 50 Uni/Hospital setting Age range = not reported Case definition = Periodontal disease: slight CAL< 3 mm moderate CAL 3 to 4 mm severe CAL ≥ 5 mm	age, sex	Intima-media thickness of the carotid artery (c-IMT) Bilateral, common (CCA), internal (ICA). Far and near wall of the vessel along a 1cm section.	PI, BOP, PD, CAL	c-IMT mean ± SD (mm) Cases 1.07 ± 0.24 Controls 0.98 ± 0.26
Southerland J.H.[599] **(2012/USA) Cross-sectional**	N = 6048 Subset of Atherosclerosis In 1996-1998 Conducted at ARIC visit 4 Risk in Communities (ARIC) Age range = 52 – 74 Case definition = Periodontitis as: % PD ≥ 4 mm and CAL ≥ 3 mm None 0%, Mild-moderate 0 – < 15%, Severe ≥15%	gender, age, race/centre, LDL and HDL cholesterol, BMI, triglycerides, hypertension, smoking, income and education.	Intima-media thickness of the carotid artery (c-IMT) Extracranial segments Bilateral, bifurcation, internal, common. The far (posterior) wall of the vessel along a 1cm section. Measurements were the mean of the six segments. When recordings of the 6 sites were missing the means at the missing sites were imputed from sex- and race specificmultivariate linear models of mean IMT as a function of age, body mass index, and arterial depth, fit by maximal likelihood methods using BMDP 5 V.	PD, CAL, REC	c-IMT ≥1 mm OR, 95% CI Related to periodontal status No diabetes Healthy 1.0 Mild-Mod 1.0 (0.8–1.2) Severe 1.2 (0.9–1.6) Diabetes Healthy 1.3 (0.8–2.9) Mild-Mod 1.4 (0.9–2.0) Severe 2.2 (1.4–3.5)
Hayashida H.[583] **(2013/Japan) Cross-sectional Included in the quantitative results**	N = 1053 Population-based Age range = not reported Case definition = No definition of periodontitis	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) Bilateral, common (CCA), Far (posterior) wall	PD, CAL	c-IMT mean ± SD (mm) Severe Periodontitis 0.94 ± 0.19 Moderate Periodontitis 0.91 ± 0.18 Mild or no Periodontitis 0.86 ± 0.16

Table 17 Characteristics of intervention studies included in the qualitative analysis, c-IMT outcomes

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Piconi S.[399] **(2009/Italy)**	N = 35 University/Hospital setting age range= 38-57 Case definition = No definition of periodontitis	Not adjusted	Intima-media thickness of the carotid artery (c-IMT) bilateral	PSR, PI, BOP	c-IMT Medians ± SD (mm) - Carotid Bifurcation Bl = 0.55 ± 0.03 6M = 0.40 ± 0.04 12M = 0.45 ± 0.04 BL vs 6M: P = 0.001 BL vs 12M: P = 0.01 - 1 cm from Bifurcation BL = 0.49 ± 0.02 6M = 0.38 ± 0.042 12M = 0.37 ± 0.03 BL vs 6M: P = 0.003 BL vs 12M: P < 0.001 - 2 cm from Bifurcation BL = 0.50 ± 0.02 6M = 0.42 ± 0.06 12M = 0.39 ± 0.03 BL vs 12M: P = 0.001

Table 18 Characteristics of observational studies included in the qualitative analysis, FMD outcomes

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Amar S.[505] **(2003/USA) Case-control Included in the quantitative results**	N = 55(Test = 26; Control = 29) Uni/Hospital settings Age range = not reported Case definition = Advanced periodontal disease 6 teeth with PD>5 mm CAL≥3 mm in 3 aspects of each involved tooth Control definition = Healthy subjects without periodontal disease recruited by advertisement	age, sex, HDL-C, systolic BP	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD) Endothelium –independent flow-mediated dilatation (EID)	PD, BOP, CAL, REC	EDD mean ± SD (%) Controls 11.7 ± 5.3 Test 7.8 ± 4.6 p = 0.005 EDD mean ± SD (mm) Controls 0.45 ± 0.16 Test 0.31 ± 0.15 p = 0.003 EID mean ± SD (%) Controls 18.9 ± 11.0 Test 16.3 ± 8.3 p = 0.37
Li P.[515] **(2011/China) Cross-sectional Included in the quantitative results**	N = 59 (MS group = 26) (non-MS = 33) Uni/Hospital setting Age Range = 23-76 (MS group) 37-71 (non-MS) Case definition = periodontal disease as: CAL >3mm: 1% to 32%= mild 33% to 66% = moderate 67% to 100% = severe	Not adjusted	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD)	PD, CAL, BI, PI, missing teeth	EDD mean ± SD (%) Non MS group No/mild PD 13.12 ± 6.57 Moderate/severe 10.43 ± 7.43 MS Group No/mild PD 9.22 ± 5.43 Moderate/severe 6.98 ± 4.89 p = 0.05

Table 18 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Ruiz A.J.[600] (2013/Colombia) Case-control Included in the quantitative results	N = 30(Test = 15; Control = 15) Uni/Hospital settings Age range = not reported Case definition = Advanced periodontal disease PD>4 mm CAL≥5 mm Control definition = subjects without periodontal disease	Not adjusted	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD)	PD, BOP, CAL,	EDD mean ± SD (%) Controls 14.8 ± 7.25 Test 13.21 ± 7 p = 0.5466

Table 19 Characteristics of intervention studies included in the qualitative analysis, FMD outcomes

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Tonetti M.[12] **(2007/UK) RCT**	N = 120 (Intensive-treatment group = 61, Control-Treatment Group = 59) Uni/Hospital settings Age range = not reported Case Definition = severe generalized periodontitis PD >6 mm and bone loss of >30% with 50% or more of teeth affected	Age, sex, smoking race, BMI, vessel diameter for EDD and EID, PD-diagnosis	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD) Endothelium –independent flow-mediated dilatation (EID)	PD, REC, BOP, FMPS	EDD mean ± SD (mm / %) BL - IPT = 3.7 ± 0.8 / 7.1 ± 4.2 - CPT = 3.6 ± 0.6 / 6.5 ± 2.6 6M - IPT = 3.7 ± 0.9 / 8.3 ± 4.2 - CPT = 3.5 ± 0.6 / 6.2 ± 2.6 6M IPT vs CPT p = 0.193 / 0.001 EID mean ± SD (mm / %) BL - IPT = 3.8 ± 0.8 / 17.8 ± 6.4 - CPT = 3.6 ± 0.7 / 17.9 ± 6.9 6M - IPT = 3.8 ± 0.9 / 15.7 ± 7.3 - CPT = 3.5 ± 0.7 / 16.3 ± 6.7 6M IPT vs CPT p = 0.107 / 0.695
Higashi Y.[375] **(2008/Japan) RCT**	N = 52 Protocol 1 (male only) 64 Protocol 2 Uni/Hospital settings Age range = not reported Case definition = Self-reported questionnaire No definition of periodontitis	Not adjusted	Forearm blood flow (FBF) of the brachial artery (Ach response)	Not reported	Protocol 1 = Response to Ach was significantly lower in periodontal patients ($p < 0.001$) FBF response to Ach was significantly higher 24 weeks after treatment ($p < 0.001$) Protocol 2 = Response to Ach was significantly lower in periodontal patients ($p < 0.001$) FBF response to Ach was significantly higher 24 weeks after treatment($p < 0.001$)

Table 19 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Higashi Y.[413] (2009/Japan) RCT	N= 101 Uni/Hospital settings Age range = not reported Case definition: Self-reported questionnaire and Chronic periodontitis as The presence of at least 2 teeth with PD ≥ 4 mm and CAL ≥ 3mm	Not adjusted	Forearm blood flow (FBF) of the brachial artery (Ach response)	PD, CAL, BOP	Response to Ach was significantly lower in periodontal patients (p < 0.001) FBF response to Ach was significantly higher 24 weeks after treatment (14.7 ± 5.2 to 20.1 ± 6.1 ml/min per 100 ml P< 0.05
Mercanoglu F.[508] (2004/Turkey) CT Included in the quantitative results	N = 54(Test = 28; Control = 26) Uni/Hospital setting Age Range = not reported Case Definition = diagnosis of chronic periodontitis was based on CAL and radiographic bone loss	Age, sex, BMI	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD) Endothelium –independent flow-mediated dilatation (EID)	PD, CAL, GI, PI	EDD mean ± SD (%) BL Controls 19 .4 ± 8.1 Final Controls 20.3±8.6 P NS BL Test 8.4 ± 4.0 Final Test 17.7±5.7 P <0.0001 EID mean ± SD (%) BL Controls 29.5 ± 10.0 Final Controls 27.1±10.8 P NS BL Test 13.3 ± 6.3 Final Test 24.9 ± 7.3 p <0.0001
Seinost G.[393] (2005/Austria) CT Included in the quantitative results	N = 61 (Test = 30; Control= 31) Uni/Hospital setting Age range = 25-50 Case definition = Severe periodontitis as involvement of at least 6 teeth with PD≥5 mm, AL ≥3 mm in 3 aspect of each involved tooth	Age, sex, smoking BMI, HDL-C	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD) Endothelium –independent flow-mediated dilatation (EID)	PD, BOP, REC	EDD mean ± SD (mm) Controls 0.29 ± 0.1 Test before Rx 0.2 ± 0.14 Test aft Rx 0.3 ± 0.14 P< 0.01 EDD mean ± SD (%) Controls 8.5 ± 3.4 Test before Rx 6.1 ± 4.4 Test aft Rx 9.8 ± 5.7 P < 0.01 EID mean ± SD (%) Controls 22.5 ± 6.7 Test before Rx 23.7 ± 3.2 Test aft Rx 23.5 ± 7.9

Table 19 continued

First Author (Year/Country) study design	Population/Sample	Covariates included for adjustment	Surrogate end-point for CVD (dependent variable)	Periodontal Status (independent variable)	Results
Elter J.R.[507] (2006/USA) **Pilot study**	N = 22 (No control group) Uni/Hospital settings and volunteer subjects. Age range = 31-55 Case definition = Moderate to severe periodontal disease defined as at least 4 sites with PD ≥ 5mm distributed in at least 2 quadrants and at least 2 of the 4 sites with AL ≥ 3 mm	Not adjusted	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD) Endothelium –independent flow-mediated dilatation (EID)	PD, AL, PI, GI	EDD (%) mean ± SD Baseline1 8.9 ± 4.7 Baseline2 8.2 ± 5.0 p for difference from BL1= .197 Pooled Baseline 8.6 ±4.7 Post-treatment 10.2 ± 3.9 p for difference from Pool BL= 0.034 EID (%) mean ± SD Baseline1 19.5 ± 7.7 Baseline 2 20.3 ± 10.6 p for difference from BL1= 0.450 Pooled baseline 19.8 ± 8.6 Post-treatment 21.3 ± 8.0 p for difference from PoolBl= .365
Blum A.[506] (41) (2007/Israel) **CT** **Included in the quantitative results**	N = 32 (Test = 22; Control= 10) Uni/Hospital setting Age Range = not reported Case Definition = Chronic periodontitis as at least 18 teeth and 1 interproximal area of PD≥ 5 mm	Not adjusted	Endothelium-dependent flow-mediated dilatation of the brachial artery (EDD) Endothelium –independent flow-mediated dilatation (EID)	PD, BOP, CAL, PI	EDD mean ± SD (%) BL Controls 16.60 ± 7.86 BL Test 4.12 ± 3.96 p = 0.0000 After Treatment 11.12 ± 7.22 p = 0.0007

3.3.3. Quantitative results

3.3.3.1. Mean c-IMT value in individuals with or without PD

7 out of 10 case-control studies comparing differences in c-IMT between individuals with and without PD could be included in a meta-analysis[476,513,585-587,591,592]. 3 publications were excluded; 2 papers reported data on the same population analyzed in previous publications[588,589], and 1 study was biased in the selection of controls[590]. 9 out of 15 cross-sectional studies[512,514-517,582-584,598] were suitable for a meta-analysis. 6 studies were excluded for the following reasons: 1 performed analysis on the same sub-sample of another included study[599], 3 related c-IMT to antibody levels of periodontal pathogens[594-596], 2 calculated c-IMT values across quintiles or tertiles of periodontal definitions[593,597]. Li P. et al. assessed c-IMT in two different samples, individuals with or without metabolic syndrome and with or without PD. We therefore considered the data as 2 separate studies[515]. In some of the included studies, individuals were divided in different groups according to PD severity. In this case the severe PD group was chosen for the analysis. The mean difference in c-IMT values in the individual studies ranged from - 0.04mm to 0.19mm. Adopting a random-effects model, a meta-analysis (4720 individuals) indicated a higher c-IMT in individuals with PD (0.08 mm 95% CI [0.07, 0.09], P = 0.000) compared to controls (Figure 20). A chi-squared test and I-squared test for heterogeneity did not show statistically significant heterogeneity (P = 0.832 and I^2 = 0.0%). Funnel-plot analysis (Figure 21 A) did not show evidence of biases using the Egger test of symmetry (P = 0.935) and sensitivity analysis did not alter the findings.

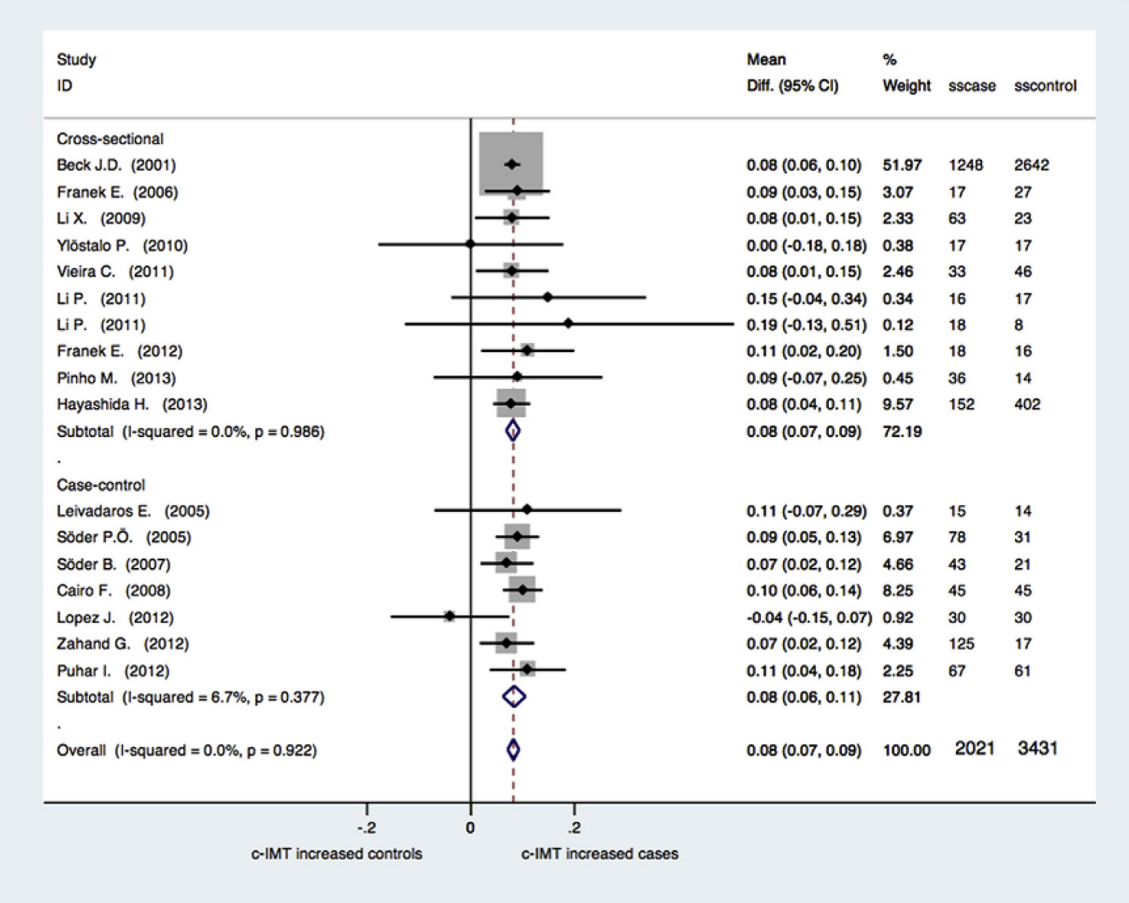


Figure 20 *Association between Periodontitis and Carotid Intima-Media Thickness (c-IMT)*

Difference of c-IMT means between individuals with periodontitis versus controls in observational studies. Horizontal lines representing 95% CI; lower diamond represents the overall effect size, random effects models. sscase sample size cases, sscontrols sample size controls

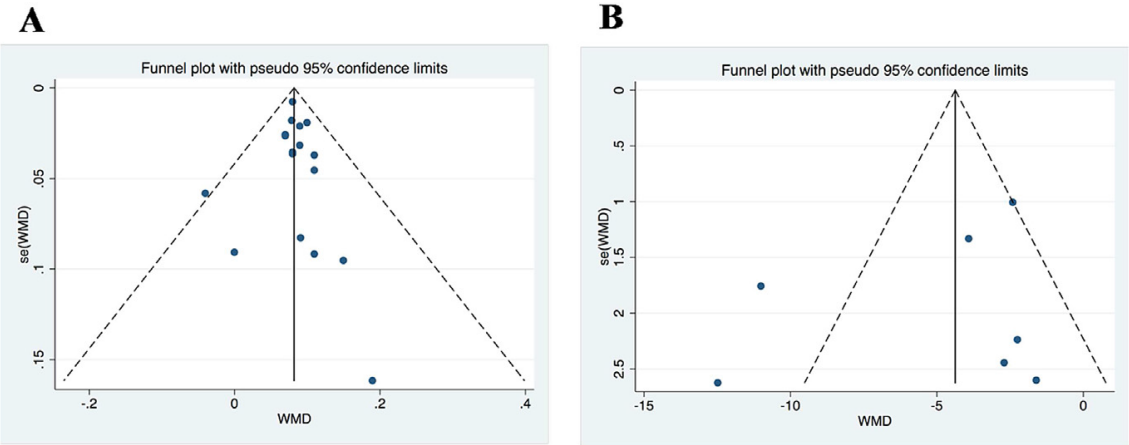


Figure 21 *Association between periodontitis, c-IMT (A) and FMD (B). Funnel plots of observational studies.*

3.3.3.2. Mean FMD values in individuals with or without PD

Differences in FMD (%) between individual with or without PD, reported by 3 observational studies[505,515,600] and baseline data from 3 controlled trials[393,506,508], were included in the meta-analysis. Individual studies mean difference in percentage of endothelial dependent dilatation (EDD) ranged from -12.48% to -2.40% with all 4 studies showing statistically significant difference. In individuals with PD, EDD was lower by 5.1% (95% CI [-8.11, -2.08], P =0.002) compared to controls (Figure 22). A chi-squared test and I-squared test for heterogeneity showed however a statistically significant heterogeneity (P = 0.000 and I^2 = 88.9%). Funnel-plot analysis (Fig. 21 B) showed a discrete asymmetry that could be an evidence of publication bias, Egger test (P = 0.076) and sensitivity analysis did not alter the findings. The funnel plot asymmetry could be probably attributed to the high heterogeneity among the studies.

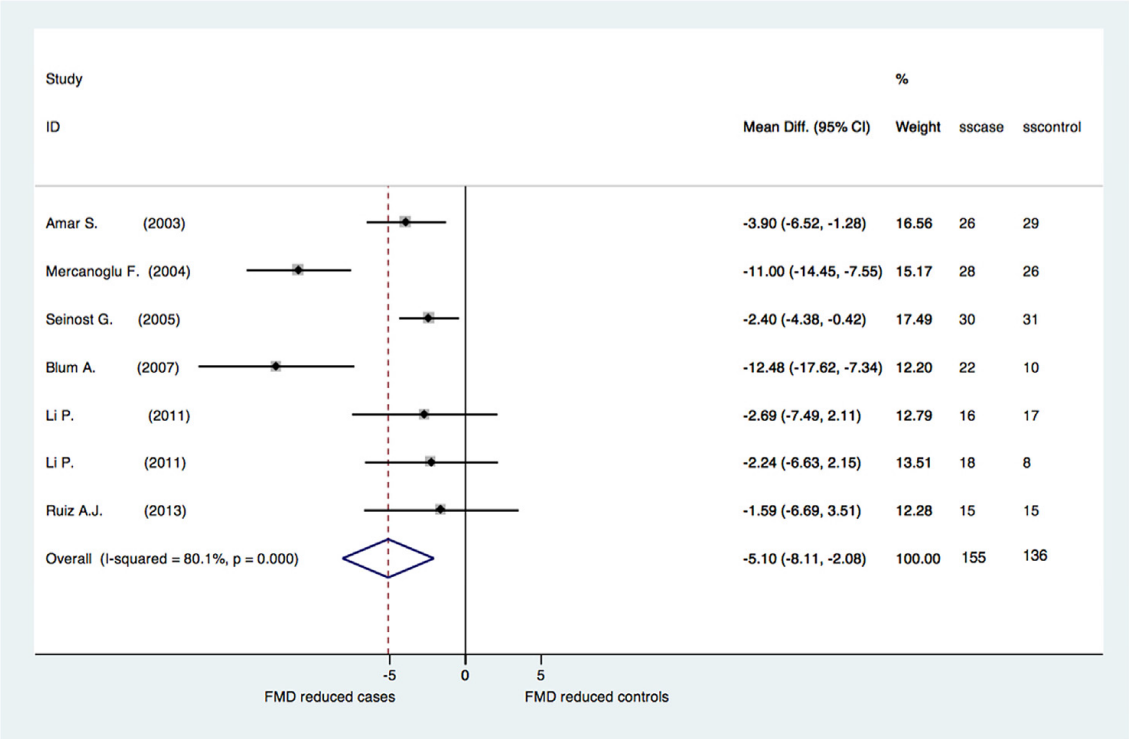


Figure 22 *Association between Periodontitis and Flow Mediated Dilatation (FMD)*

Difference of the FMD means between individuals with periodontitis versus controls in observational studies. Horizontal lines representing 95% CI; diamond represents the overall effect size, random effects models. sscase sample size cases, sscontrols sample size controls.

3.3.3.3. Effect of periodontal therapy on EDD

It was possible to analyze the summary difference in mean FMD (%) between test and control after periodontal treatment in 3 of 6 controlled trials[393,506,508] (147 individuals) (Fig. 23). Mean improvement in FMD after periodontal intervention ranged from to 3.7 to 9.3%. The overall summary estimate of the mean increase in FMD based on a random-effects model was 6.64% (95% CI [2.83 to 10.44], $p < 0.0001$). A chi-squared test for heterogeneity and the I-squared index showed statistically significant heterogeneity ($p = 0.011$ and $I^2 = 78\%$). The Egger test didn't show a small study effect ($p = 0.956$) and sensitivity analysis identified two influential studies potentially related to the small number of included trials.

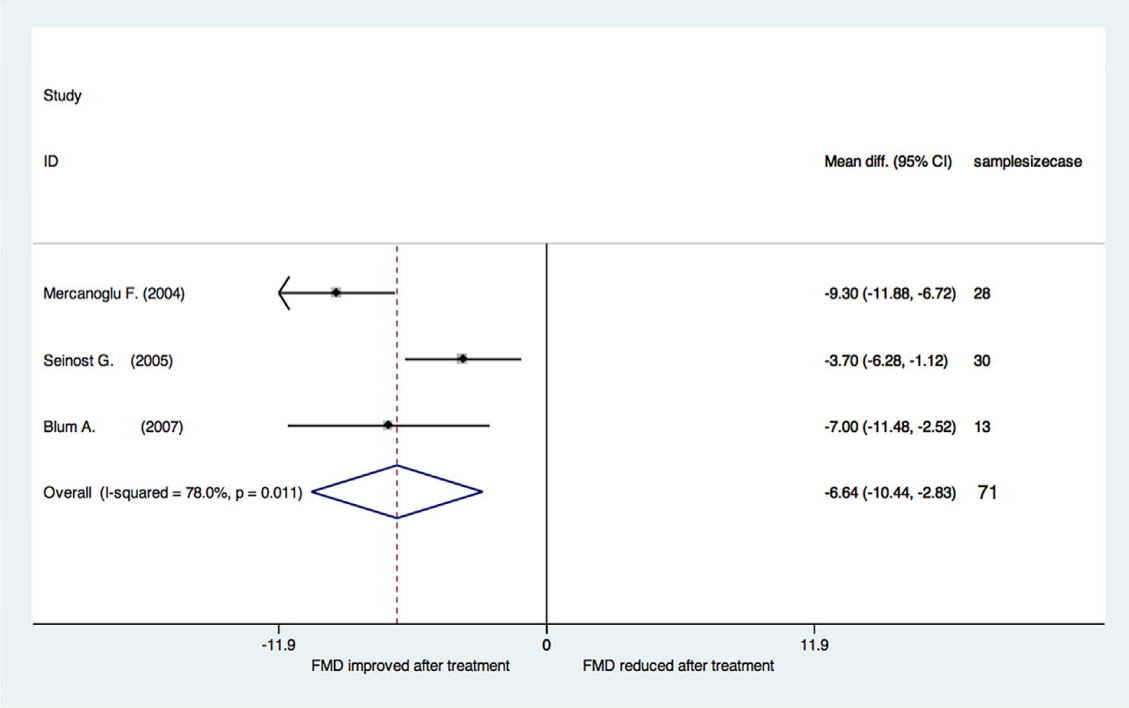


Figure 23 *Association between Periodontal Treatment and Flow Mediated Dilatation (FMD)*

Difference of the FMD means before and after periodontal treatment in controlled clinical trials. Horizontal lines representing 95% CI; diamond represents the overall effect size, random effects models. sscase sample size cases, sscontrols sample size controls.

3.4. Discussion

Our findings indicate that PD is associated with greater c-IMT and impaired FMD and that periodontal treatment improves endothelial function. Despite the number of reports on the topic, this is the first systematic review and meta-analysis linking PD to specific CVD surrogate outcomes in the general population. These associations further reiterate the importance of oral health as a possible un-recognized common factor building on the individual cardiovascular risk. Our analysis relates to c-IMT measurements from 4720 individuals showing that individuals with PD have on average a greater c-IMT (0.08 mm) when compared with individuals without PD. Despite the small magnitude of this difference, current evidence suggests that in the general population the mean estimates of c-IMT progression ranged from 0.001 to 0.030 mm per year for mean common carotid artery intima-media thickness[601]. Individuals with PD therefore present with greater burden of subclinical atherosclerosis compared to controls. This finding is consistent with a previous report from our group demonstrating in a case-control study that individuals with PD present with shorter leucocytes telomere length independent of age differences and other common confounders[602]. Shorter telomere length has been linked to increased mortality and greater progression of other common chronic diseases (i.e. CVD and diabetes) implying faster aging processes in people with PD[603]. A “chronic” exposure to a local and systemic state of inflammation and oxidative stress like those reported in people with PD could represent one of the possible mechanisms explaining this association. A number of factors however should be taken into account when interpreting collectively these associations. Indeed evidence suggests that per every 0.1 mm difference in c-IMT, the relative risk (RR) of future myocardial infarction increases to 1.15 (95% CI, 1.12 to 1.17) and to 1.18 (95% CI, 1.16 to 1.21) for stroke[604].

A number of traditional cardiovascular risk factors are all linked to this rate of progression in the following order of importance: age, gender, systolic blood pressure, HDL cholesterol, smoking, diabetes, hypertension treatment, and total cholesterol. Clearly PD shares most of these risk factors and therefore it is difficult to discern the potential impact of PD alone on the progression of c-IMT. The lack of a uniform adjustment for all these confounders in all the studies included in our meta-analysis could have also reduced the accuracy of our results. Increased values of c-IMT can truly predict cardiovascular events (CVEs). A c-IMT value of 0.9 mm is considered for instance a conservative estimate of pre-existing abnormalities according to the recent Guidelines for the management of arterial hypertension[605]; but it is still uncertain whether c-IMT assessment improves individual risk assessment in primary CVD prevention. Furthermore, it is no clear which carotid segment would provide the most relevant information (common carotid, bifurcation, internal carotid arteries) as well as which summary measure should be adopted (mean, maximal, mean of the maximal). Studies aimed at evaluating whether ultrasound measures add prognostic information to traditional vascular risk factors (VRFs), reported different conclusions[89,527,606-608]. Only few of them showed a null or modest usefulness of c-IMT measurements in a general population[89,606,607] while results for the Rotterdam Study proved its utility in the risk assessment in women only[608]. In the IMPROVE study, Baldassarre et al. examined a population with high CVD risk (elderly with ≥ 3 VRFs) and explored the net reclassification improvement obtained with the addition to the Framingham risk score (FRS) of composite c-IMT variables, rather than common c-IMT alone. Both approaches improved the reclassification with the higher changes (14.5% individuals moved from intermediate to a high risk category) obtained when using intra-adventitial common carotid diameter and the value of c-IMT min-max[527]. Despite the low level of statistical

heterogeneity detected in our analyses (I-squared 0%) the robustness of our findings could be affected by differences in the research protocols (i.e. clinical heterogeneity). The diagnosis of PD and c-IMT assessment criteria varied considerably throughout the studies included. PD was assessed using different criteria ranging from validated clinical measures such as CAL and PPD to self-defined criteria showing the lack of a uniform definition of PD[609,610]. In addition we could not group individuals with PD according to disease severity (mild, moderate and severe) as in the majority of the studies retrieved such differentiation was not performed. We therefore followed a conservative approach by comparing healthy versus severe PD individuals. This approach could have resulted in an underestimation of the reported associations. Current evidence does not allow understanding whether the higher CV risk is associated with diagnosis of PD or its severe forms only. As the prevalence of PD is relatively high in the general population, the identification of subgroups of individuals with PD at increased risk for CVD, would help in reducing possible costs associated with large scale screening. For instance, a difference of 0.08 mm would not be crucial in healthy and young individuals with PD as their absolute c-IMT would be far from the 0.9 mm value considered abnormal following the current guidelines for CV risk assessment. Specifically to c-IMT we identified multiple sources of heterogeneity such as details of the ultrasound protocol, definition of the carotid segment analysed, the assessment based on the near or far wall or both and whether c-IMT was measured only on 1 side or on both sides. A recent consensus paper recommends measurement of c-IMT in plaque-free areas[611] and we did not find explicit statements about this methodological aspect in any of the studies we analyzed. The recent publication of guidelines to assess c-IMT is likely to improve consistency of the technique in future studies. Based on our research strategy, we identified only one longitudinal study

evaluating the impact of periodontal treatment on c-IMT in people with mild/moderate PD. 6 and 12 months following periodontal treatment, a statistically significant reduction in c-IMT compared to the BL measurements was reported[399]. These findings could support a potential causal association between PD and subclinical atherosclerosis assessed by c-IMT. However considering the absence of a control group in the study, inclusion of CVD-free individuals and the lack of multiple measurements of the carotid thickness, the statement about causality is not supported by the evidence reviewed. Further clinical studies should be performed and similar findings should be confirmed in larger, controlled and possibly high-risk population (e.g. greater c-IMT). The relationship between FMD of the brachial artery and PD was also an objective of the present review. While c-IMT is a measure of sub-clinical atherosclerosis, FMD is related to the pathogenesis of atheroma and provides information regarding the early stage of its evolution. Endothelial function and specifically FMD can also predict future CVD outcomes, a recent meta-analysis reported that per every point decrease in FMD, future CVD risk increased of 10%[612]. Demonstrating that PD, due to its chronic inflammatory nature, could contribute to atheroma formation would provide important biological information useful in the prevention of endothelial dysfunction. The meta-analysis gathered data from baseline measurements of 3 controlled trials[393,506,508] and one case-control study[505] showing a mean difference of 5.1% in FMD in individuals with PD compared to controls and a mean increase of 6.64% following periodontal treatment. Our choice of including only 3 controlled trials was based on the fact that the study design and the methodology in assessing FMD differed considerably among the rest of the trials. Indeed FMD values varied considerably from study to study, with an average baseline FMD ranging from 4.1% to 8.4%. Further to this, sample characteristics such as underlying health status

and ethnicity were different and follow-up time in the studies ranged from 6 weeks to 3 months. All these differences between the studies should be considered when interpreting the data. Lastly, assessment of endothelial function with FMD is not free from methodological, physiological and technical factors that can influence the validity, reproducibility and interpretation of results in clinical research[613]. As confirmation of our cautious interpretation of the evidence, a statistically significant high level of heterogeneity was found (80.1% for case-control and 78% for intervention trials). Further evidence supporting the beneficial role of periodontal treatment in improving endothelial function derives from other 3 RCT; two were conducted assessing endothelial function with a more invasive technique (response of brachial blood flow to acetylcholine injection) and both showed a statistically significant improvement after periodontal therapy[375,413]. The third one is the only RCT reporting on measure of endothelial function with FMD. Our group concluded that 6 months after intensive periodontal therapy (associated with a substantial improvement in the gingival condition) individuals presented with a 2.0% greater value of FMD (95% CI 1.2 to 2.8; P<0.001) compared to controls[12]. Our interpretation of these studies results and the meta-analysis collectively supports a causal association between PD and endothelial dysfunction. Further research should be performed in understanding the exact mechanisms behind this link.

3.5. Conclusions

We conclude that PD is associated with greater subclinical atherosclerosis as assessed an increased c-IMT which is considered an independent predictor of CV events in high-risk populations. We also found evidence of an impaired FMD in individuals suffering from PD. Our data also suggest a beneficial effect of periodontal intervention on this

marker. Robust prospective studies are needed to improve understanding of the mechanisms underlying this association. Furthermore, intervention trials will help the identification of common pathways explaining the link between evolution of atherosclerosis and PD. Standardized protocols to measure c-IMT and FMD as well as an universally-accepted definition of PD would reduce the limitations of current findings.

4. STUDY 2: MITOCHONDRIAL OXIDATIVE STRESS, SYSTEMIC INFLAMMATION, ENDOTHELIAL FUNCTION AND METABOLIC CONTROL IN PATIENTS WITH TYPE II DIABETES MELLITUS AND PERIODONTITIS

4.1. INTRODUCTION

4.1.1. Mitochondrial dysfunction and oxidative stress: the missing link between inflammation and cardiovascular disease

It is now accepted that inflammation plays a key role in the appearance and evolution of atherosclerosis[107]. Immune cells dominate early atherosclerotic plaques, their effector molecules accelerate atheroma progression and inflammatory activation elicits acute coronary syndromes and clinical events[293]. Loss of normal endothelial function (endothelial dysfunction) is one of the earliest manifestations of inflammation-related vascular damage[80]. It is characterized by reduced bioavailability of nitric oxide (NO) and increased expression of adhesion molecules and inflammatory genes by endothelial cells[614]. We have previously shown that this early stage of vascular damage is partially reversible by modulating the levels of inflammatory exposure[12]. Using a chronic inflammatory model (periodontitis and its treatment) we have demonstrated that effective treatment of oral and systemic inflammation results in an improvement of endothelial function. While this study represented the first evidence in humans of the crucial role of systemic inflammation in attenuating the development of abnormal vascular biology, the molecular mechanisms accounting for these results have not been fully elucidated. Animal models and in vitro studies have

shown that an overproduction of reactive oxygen species (ROS) is an important mechanism by which inflammation drives CVD[615]. ROS convert native low-density lipoprotein (LDL) cholesterol to oxidised LDL cholesterol (oxLDL), highly cytotoxic molecules that reduce the anti-atherosclerotic functions of the endothelium[616]. Moreover, oxidative stress reduces the bioavailability of endothelial NO and increases vascular endothelial permeability, promoting leukocyte migration into the sub-intima space[617]. Activated inflammatory cells in the sub-intimal space produce high amounts of ROS, further increasing the oxidation of LDL and subsequent activation of local endothelial cells. Of the many potential cellular sources of chronic ROS production, mitochondria represent the most relevant under physiological conditions[618]. Mitochondrial respiration represents the mechanism by which cells consume nutrients (i.e. glucose) and oxygen to provide energy (ATP) for many cellular reactions. This process requires the presence of an electromotive differential across the inner mitochondrial membrane and is maintained by the highly coordinated activity of several enzymes (respiratory chain). Due to exogenous environmental changes (i.e. inflammation), the synchronised activity of the respiratory chain enzymes can be lost (mitochondrial dysfunction), resulting in reduced trans-membrane potential, increased ROS generation and reduced metabolic efficiency[619]. Therefore, the primary marker of mitochondrial dysfunction is the breakdown of mitochondrial membrane potential, often associated with mitochondrial DNA damage (mtDNA). Emerging evidence now suggests that increased levels of mitochondrial ROS production directly contribute to the evolution of the inflammatory-related CVD. Accumulation of mtDNA damage, and progressive respiratory chain dysfunction have been associated with atherosclerosis or cardiomyopathy in both human investigations and animal models of oxidative stress[620,621]. Moreover, major precursors of atherosclerosis -hypercholesterolemia,

hyperglycemia, hypertriglyceridemia, and even the process of aging- all induce mitochondrial dysfunction[622]. Chronic overproduction of mitochondrial ROS leads to increased oxidation of LDL and dysfunction of endothelial cells and ultimately promoting atherogenesis[623]. Much of what is known regarding the impact of mitochondrial dysfunction in CVD has been generated from animal models and *in vitro* studies.

4.1.2. Oxidative stress and mitochondrial dysfunction can account for the increased CVD risk of patients with type 2 diabetes and periodontitis

We have selected two common diseases in the general population, type 2 diabetes and periodontitis, which often coexist in the same patient and are characterized by high levels of inflammatory exposure, mitochondrial dysfunction and high risk of CVD[624]. Diabetes is a systemic disease characterized by reduced insulin activity and glucose consumption in peripheral tissues. It affects more than 150 million people worldwide, and it is estimated that this number will increase to 299 million by the year 2025[625]. CV mortality is two- to eight-folds higher in subjects with diabetes and the generation of ROS may play an important role in the aetiology of diabetic vascular complications[626,627]. Indeed, exposure of endothelial cells to high glucose levels increases ROS production, quenching endothelial derived NO and reducing the anti-atherosclerotic properties of the quiescent endothelium[628]. Multiple studies have documented that human insulin resistance is accompanied by impaired *in vivo* mitochondrial oxidative function[629,630]. The development of mitochondrial dysfunction could represent a crucial mechanism, linking hyperglycaemic-induced oxidative stress production and the development of vascular damage in patients with diabetes. Periodontitis is currently considered an important global oral health burden together with dental caries; its reported prevalence ranges from 20% to 50% in the general

population[166,631,632]. The local chronic inflammatory response to the oral bacteria is associated with systemic inflammation[633,634] and increased future risk of other co-morbidities including cardiovascular diseases[635]. Oxidative stress and mitochondrial dysfunction may play a central role in the association between periodontitis and CVD. Indeed, an excess of ROS coupled with a decreased antioxidant capacity have been already documented in the peripheral blood of patients suffering from periodontitis [497]. Similarly, mitochondrial dysfunction has been described in the peripheral blood mononuclear cells and gingival tissues of subjects affected by chronic periodontitis[636,637].

In patients suffering from type 2 diabetes and periodontitis, the systemic inflammatory response associated with the oral disease is linked with impaired gluco-metabolic control[185] and increases CV mortality[321]. There is also evidence that treatment of PD could improve glucose management in patients with diabetes[638] and improve endothelial function[12]. As the biological pathways accounting for these findings are poorly understood, our group has established and validated a novel experimental model to examine the complex mechanisms behind vascular adaptations to acute and chronic inflammatory responses: PD treatment model. The primary aim of this project was to determine whether, in patients with periodontitis and type 2 diabetes, endothelial function can be improved by reducing local and systemic inflammation after periodontal treatment. The secondary aim was to explore whether mitochondrial function correlated with measures of endothelial function and gluco-metabolic control.

4.2. METHODS

4.2.1. Study Design

We have analyzed changes of mtROS production and mitochondrial function in a parallel group, single-blind, randomized, controlled trial which evaluated the effect of periodontal therapy on glycemic control in T2DM.

Recruitment and study flowcharts are reported in Figure 24.

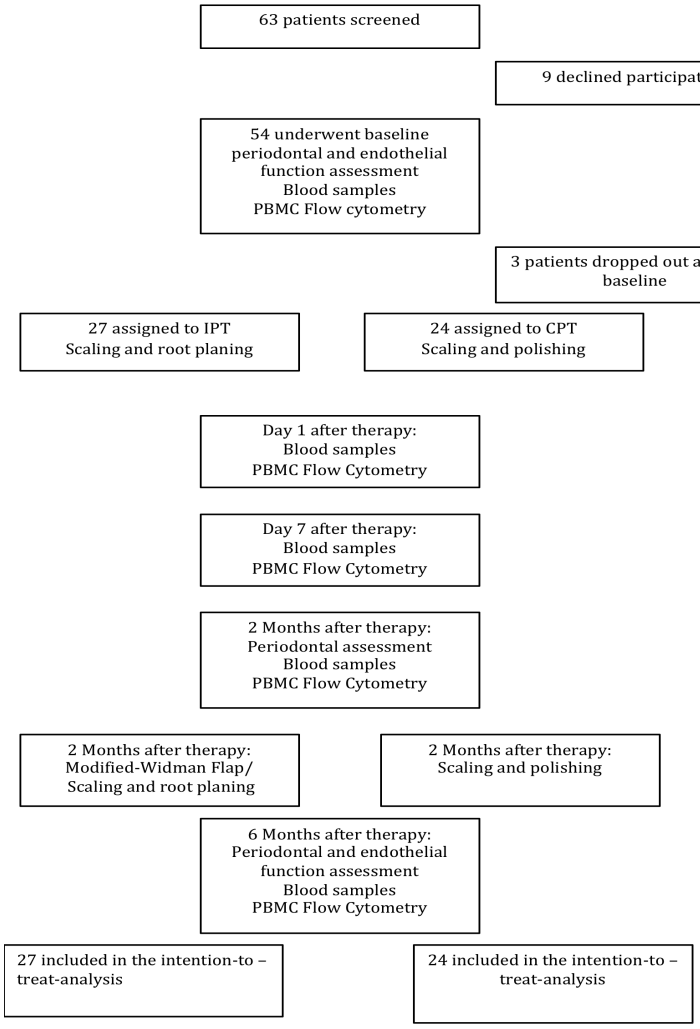


Figure 24 *Study Flowchart*

Consecutive patients with T2DM and moderate to severe PD referred to the Eastman Dental Hospital (London, United Kingdom) between December 2011 and September 2012 fulfilling the study requirements were included in our sample.

The inclusion criteria were:

aged over 18 years, diagnosis of T2DM, a minimum of 15 teeth, at least 20 sites with periodontal pocket depth (PPD) ≥ 5mm.

The exclusion criteria were:

pregnancy/lactation, HIV or Hepatitis (B, C), subjects with uncontrolled systemic diseases or neoplasm, chronic antibiotic therapy or requiring antibiotic coverage for dental procedures, chronic treatment with medications known to affect periodontal status (phenytoin, cyclosporine).

Following a baseline visit, each participant was randomly allocated, with the use of a computer-generated table, to receive either intensive periodontal therapy (IPT) or control periodontal therapy (CPT). Blood samples were collected at baseline and 6 months after the periodontal therapy for PBMC isolation and assessment of mitochondrial parameters, as well as to assay levels of inflammatory cytokines and cardiovascular risk factors. Endothelial function was assessed by flow-mediated dilation (FMD) at baseline and 6 months from treatment. Laboratory and vascular technicians were blind to the patient treatment allocation. All patients gave written informed consent. The study was approved by the local ethics committee (Ref 07/H0714/97, joint UCL/UCLH Committees on Ethics of Human Research, Committee A).

4.2.2. Periodontal Examination and Therapy

Periodontal data were recorded at baseline and 2 months and 6 months after the therapy. The data included the PPD and the recession of the gingival margin relative to

the cement-enamel junction at six sites per tooth. The presence or absence of supragingival dental plaque and gingival bleeding on probing was also recorded. Oral hygiene instructions were given to all patients. The IPT arm underwent extraction of hopeless teeth, full mouth scaling and root planing (SRP) under local anaesthesia within a single session. As described in Chapter 2, corrective periodontal therapy was also performed in patients with adequate control of dental plaque in deeper periodontal sites and non-surgical treatment repeated in all remaining sites (9 participants). In the CPT arm patients had a repeated cycle of supra-gingival scaling and polishing.

4.2.3. Mitochondrial membrane potential and ROS production

Peripheral blood mononuclear cells (PBMC) were isolated following standard procedures by density gradient centrifugation with Ficoll (Ficoll-Paque PLUS, GE, UK) from an aliquot of heparinised blood collected at each study visit. Mitochondrial oxidative stress production and membrane potential were assessed by flow cytometry using the mito probe MitoSOX Red (Invitrogen, UK) and 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen), respectively as described in previously described in the Methods section of this thesis.

4.2.4. LPS assay

Serum endotoxin activity was determined by the Limulus Amebosyte Lysate test kit with a chromogenic substrate (Lonza, Walkersville, MD, US). The steps of the LPS assay are described in the Methods chapter. Intra- and inter- coefficient of variations for all assays were < 5%.

4.2.5. Endothelial function

Endothelium-dependent vasodilatation of the brachial artery at baseline and 6 months follow up was assessed by means of ultrasound imaging (Acuson XP 128/10, Siemens) with the use of a 7-MHz linear probe and automated vessel-diameter measurements (Brachial Tools, version 3.2.6, Medical Imaging Applications), as previously described. A single examiner blind to the patient treatment allocation acquired the images of the brachial artery in the morning, while patients were fasting, in a temperature-controlled room after 10 minutes of rest. Endothelium-independent dilatation was measured after sublingual administration of 25 μg of nitroglycerin, according to the same recording protocol.

4.2.6. Inflammatory, metabolic and vascular injury assays

Serum and plasma were assayed for a variety of inflammatory biomarkers at the baseline and 6 months visit. Sample were centrifuged within 1 hour of collection and stored at –70°C for future analysis. The circulating inflammatory markers assessed were: IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α (Mesoscale, Human pro-inflammatory 7-plex) and sICAM-3, E-Selectin, P-Selectin, Thrombomodulin (Mesoscale, Human vascular injury Panel I) serum levels. HbA1c and insulin levels were assayed on an automated analyzer (Cobas 8000 analyzer, Roche Diagnostics Corp). All samples were evaluated in a blinded fashion and, to minimize variability, all assays were performed at the end of the study, in duplicates and on the same ELISA plate at both time points for a given patient.

4.2.7. Statistical Analysis

As there were no data in the literature that allow predicting changes of mitochondrial function after anti-inflammatory treatment, we based the sample size calculation on

the changes of measures of inflammation and cardiovascular health expected after dental treatment. A total of 44 patients (22 per study arm) provided 90% power (2 sided, p=0.05) to detect a 2% difference in a common measure of vascular function (flow-mediated dilatation) between groups using an estimated standard deviation of 1.6 derived from our previous published data. Therefore the sample size of the clinical study included 24 participants per group (10% drop out rate).

All data produced by the laboratory analyses were entered in a computer database proofed for entry errors and loaded in the appropriate software for analysis (SPSS ver21). Changes in mitochondrial function were analyzed by analysis of variance for repeated measures between IPT and CPT groups including data at baseline, day 1, day 7, 2 months and 6 months. A conservative F-test was used to interpret the model using the Greenhouse-Geisser correction to account for compound symmetry violations. Changes in inflammatory biomarkers were correlated with changes in mitochondrial membrane potential and ROS production at day 1 and 6 months. The relative changes were calculated by subtracting the value at day 1 or 6 months from the baseline value of the outcome of interest. These changes were then analyzed both with simple Pearson or Spearman tests (depending on the data distribution) and multiple linear regression analyses with robust standard errors. Univariate analyses were performed with crude values of each outcome variable between study groups at different time points. Further multivariate models were constructed including variables that showed a statistically significant association with the outcome from univariate analyses and they included age, gender, smoking, body weight and ethnicity. Significance was set to be at $p < 0.05$.

4.3. RESULTS

Between December 2011 and September 2012 we screened 63 suitable participants: 9 declined the participation therefore 54 were included in the study. After randomization 27 patients were allocated to each treatment group. Three participants in the CPT group refused to provide additional blood for the mitochondrial assays following the baseline visit. All participants completed the trial. There were no major adverse events reported in both groups.

4.3.1. Patient characteristics

At baseline patients in the control-treatment group and the intensive-treatment group had similar characteristics including age, gender, smoking status, lipid levels, body-mass index, blood glucose levels (Table 20) and medication use (Table 21).

Table 20 Baseline characteristics of the study participants

Variable (mean±SD)	CPT (N=24)	IPT (M=27)
Age, years	58±11	56±9
BMI, Kg/m^2	32±5	32±7
Gender, Males	10(42%)	15(56%)
Smoking, Current	1(4%)	1(4%)
Systolic BP, mmHg	134±19	136±18
Diastolic BP, mmHg	81±11	84±11
HbA1c (%)	7.7±1.2	7.9±1.4
Cholesterol, mmol/l	4.3±1.0	4.3±1.1
HDL, mmol/l	1.3±0.4	1.3±0.4
LDL, mmol/l	2.0±0.9	2.3±0.9
FMD%	4.18±2.28	4.13±2.98
CRP*, mg/l	1.8 (3.1)	2.2 (3.0)
TNF-α*, pg/ml	3.7 (1.8)	4.0 (1.7)
s-Eselectin*, pg/ml	24.8(20.2)	25.8(11.0)
s-Pselectin*, pg/ml	118.8(35.8)	103.1(30.1)

Interferon-γ*, pg/ml	1.1(2.4)	0.9(1.9)
Mitosox, MFI	25.4±12.5	23.4±10.5
JC-1, MFI	5.33±3.98	5.84±5.02
LPS, EU/mL	0.39±1.49	0.27±1.74

*Values are expressed as means±SD or *median (interquartile range) for non-normally distributed variables.*

Table 21 Baseline medication use

Variable (mean±SD)	CPT (N=24)	IPT (M=27)
Biguanides	20(84.2%)	26(96.3%)
Sulfonylureas	11(47.4%)	13(48.1%)
Thiazolidinediones Pioglitazone	4(15.8%)	1(3.7%)
DPP-4 Inhibitors	5(21.1%)	4(14.8%)
Incretins GLP-1 analogs	0(0.0%)	4(14.8%)
Betablocker	8(31.6%)	6(22.2%)
Diuretic	5(21.1%)	4(14.8%)
Ca channel blocker	4(15.8%)	5(18.5%)
Alpha blocker	1(5.3%)	3(11.1%)
Angiotensin-II blocker	3(10.5%)	5(18.5%)
Ace Inhibitors	13(52.6%)	12(44.4%)
Statin	19(78.9%)	22(81.5%)
Aspirin	11(47.4%)	7(25.9%)

4.3.2. Periodontal outcomes

A statistically significant interaction between treatment and time ($p<0.001$) was detected with repeated-measures ANOVA of the periodontal outcomes. The intensive-treatment group had lower scores for plaque 6 months after therapy (absolute difference of 27%; 95% CI, 15-40; $p<0.001$) compared to the control group (Table 22). Similarly patients in the intensive-treatment group had fewer periodontal pockets 6

months after therapy (average difference between groups of 15; 95% CI, 3-26; p=0.014) (Table 22).

Table 22 Periodontal parameters at Baseline and 6 Months after Periodontal Therapy

Variable	Group	Baseline	6 months
FMPS, % §	IPT	80±13	42±20 †
	CPT	82±15	69±20
FMBS, % ¶	IPT	70±20	36±21 †
	CPT	72±15	58±18
PPD, mm	IPT	3.9±0.8	2.9±0.7
	CPT	3.6±0.7	3.3±0.7
REC, mm	IPT	1.2±0.9	1.7±1.0
	CPT	1.3±0.8	1.6±0.8
NPKTs, n *	IPT	56±28	17±16 †
	CPT	41±19	32±22

Values are expressed as means±SD.
† P<0.001 for the comparison with the standard-treatment group.
IPT: intensive treatment group, CPT: control treatment group, FMPS: full mouth plaque score, FMBS: full mouth bleeding score, PPD: periodontal pocket depth, REC: gingival recession, NPKTS, number of pockets.
¶ Scores for full-mouth gingival bleeding were calculated for each patient as the number of sites with gingival bleeding on probing divided by the total number of sites per mouth, multiplied by 100.
§ Scores for full-mouth plaque were calculated for each patient as the number of sites with detectable plaque divided by the total number of sites per mouth, multiplied by 100.
** Periodontal pockets identified as sites with probing greater or equal to 5 mm*

4.3.3. Mitochondrial ROS production and Membrane potential

The PBMC of the IPT group had a significantly lower mtROS production compared to the CPT group at 6 months following dental treatment (unadjusted difference of 8.49; 95% CI, 1.31-15.68; p=0.02) (Figure 25 A). When subpopulations of PBMC were analysed separately, the reduced mtROS generation of PBMC was mainly due to a reduction in oxidative stress production in lymphocytes (unadjusted difference of 6.32;

95% CI, 0.51-12.14; p=0.034), although a non-statistically significant trend was detected also in monocytes (unadjusted difference of 14.28; 95% CI, -28.26-56.81; p=0.501) (figure 25 B, 25 C). There was no difference between groups in the mitochondrial membrane potential of PBMC(unadjusted difference of -0.38; 95% CI,-1.0-0.23; p=0.223), lymphocytes (unadjusted difference of -0.13; 95% CI, -0.76-0.51; p=0.692) and macrophages (unadjusted difference of 0.19; 95% CI, -0.28-0.65; p=0.423) (Figure 26A, 26B, 26C).

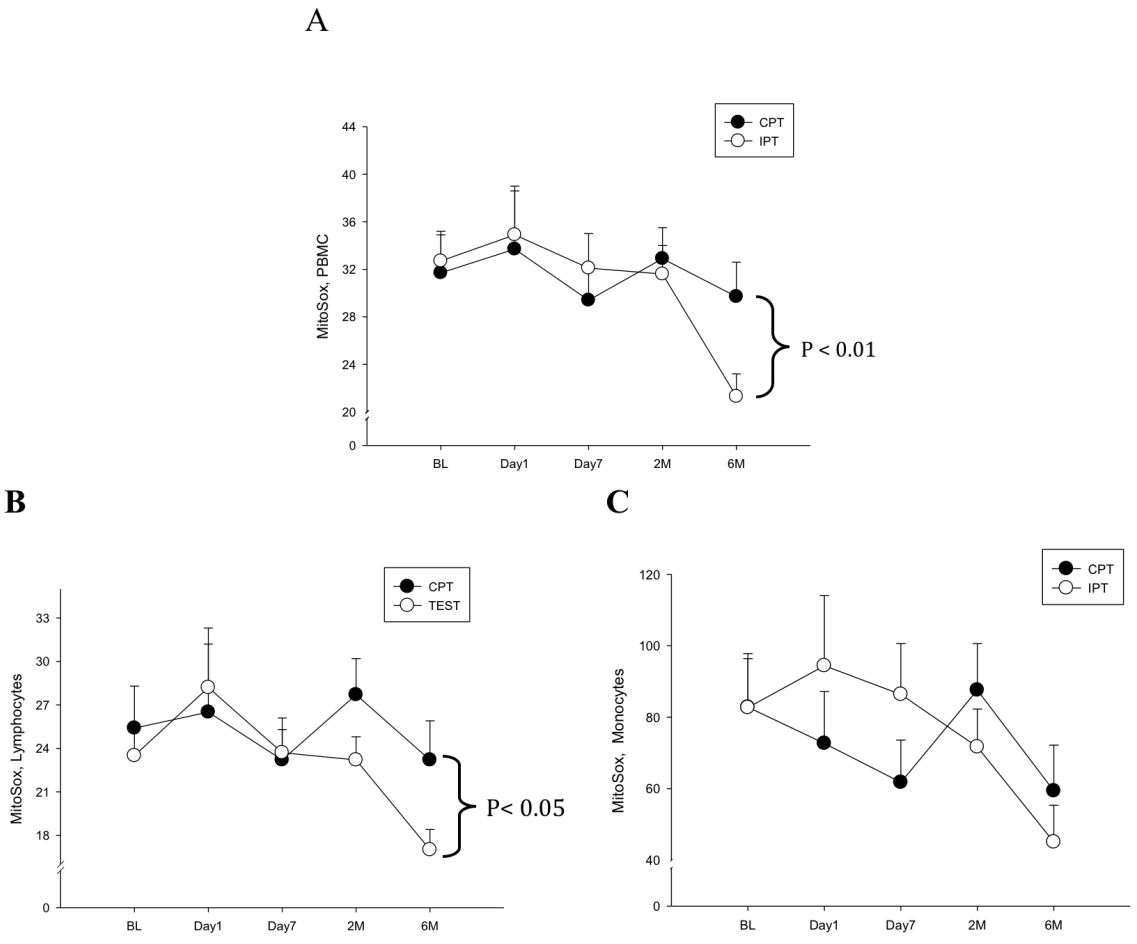


Figure 25 *Changes in mtROS production during the study period.*

A) PBMC, B) Lymphocytes, C) Monocytes. I bars represent SE. mtROS production was significantly lower in PBMC (P<0.01) and lymphocytes (P<0.05) at 6 month in the IPT compared to the CPT group. Data adjusted for age, gender, smoking, body weight and ethnicity.

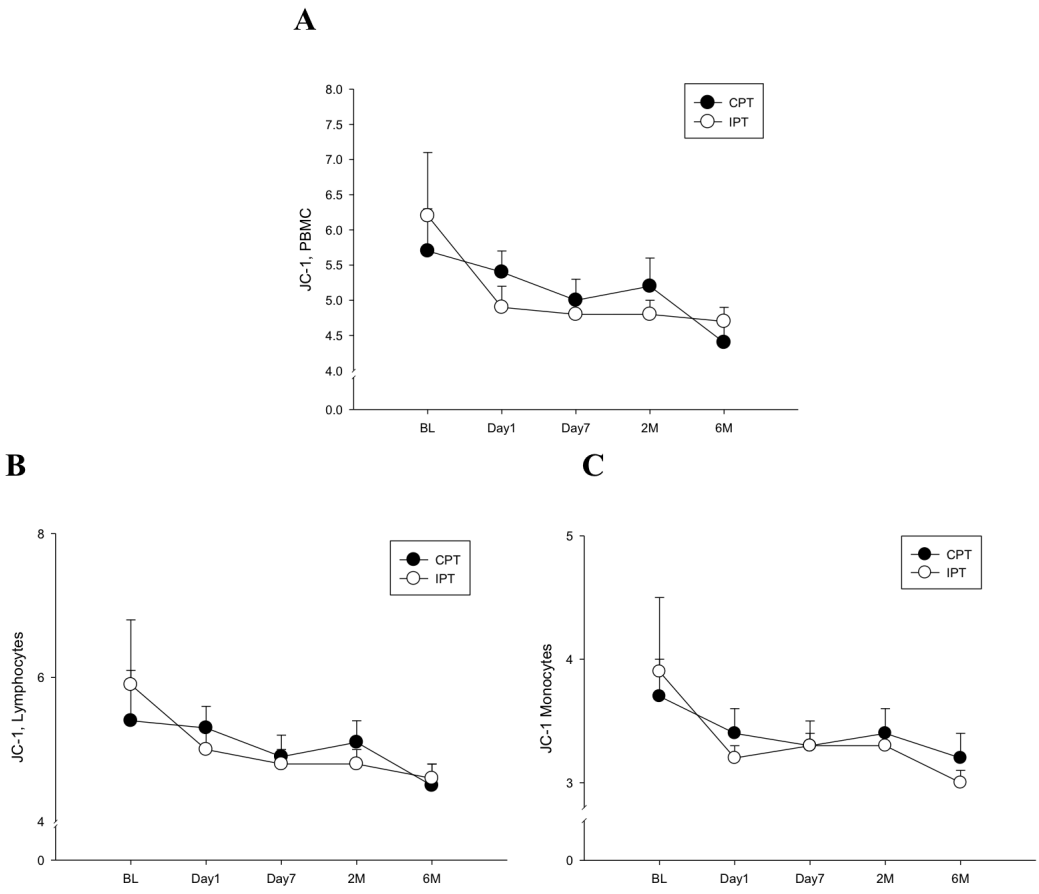


Figure 26 *Changes in JC-1 during the study period.*

PBMC (A), Lymphocytes (B), Monocytes (C). There were no differences between IPT and CPT groups. I bars represent SE.

4.3.4. LPS

A statistically significant difference in LPS plasma levels between CPT and IPT patients was observed 6 months after periodontal treatment (unadjusted difference of 2.73 ; 95% CI, 1.15-6.45; p=0.023) (Figure 27).

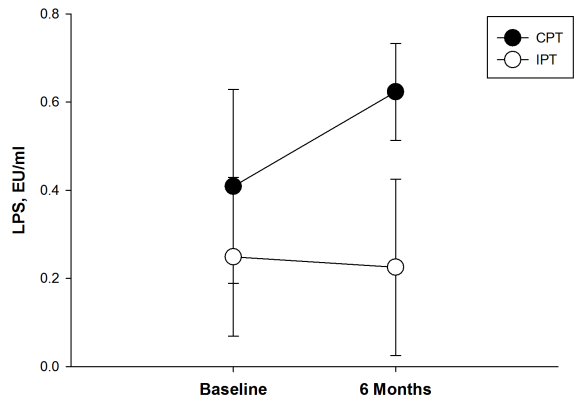


Figure 27 *Changes in LPS during the study period*

4.3.5. Vascular function

There was a significant interaction between treatment and time using repeated-measures analysis of variance of flow-mediated dilatation ($p<0.001$) (Fig. 28). After 6 months from treatment, FMD was higher in the intensive treatment group than in the control-treatment group (absolute difference of 0.9%; 95% CI, 0.3-1.4; $p=0.002$). There was an average increase in FMD from baseline of 24.0±10.7% in the IPT group compared to CPT ($p=0.03$), (Figure 29). Conversely, no interaction between treatment and time was observed for nitroglycerin-dependent dilatation or for brachial artery diameters and no changes of the nitroglycerin-dependent dilatation were observed from baseline to 6 months (unadjusted difference of 0.3; 95% CI, -1.5-2.0; $p=0.65$).

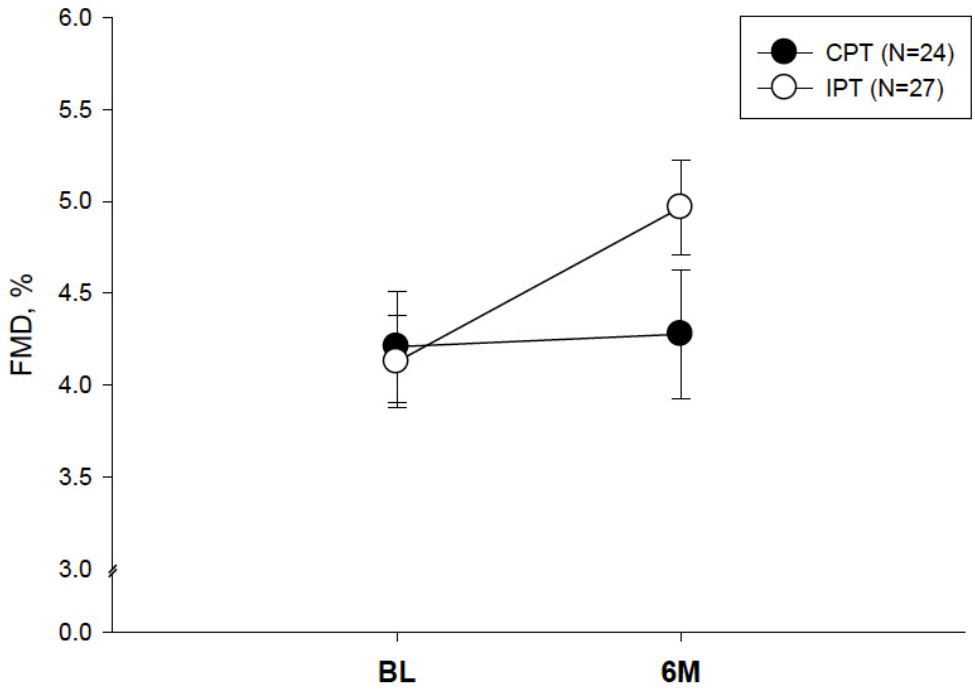


Figure 28 *Flow mediated dilatation during the study duration*

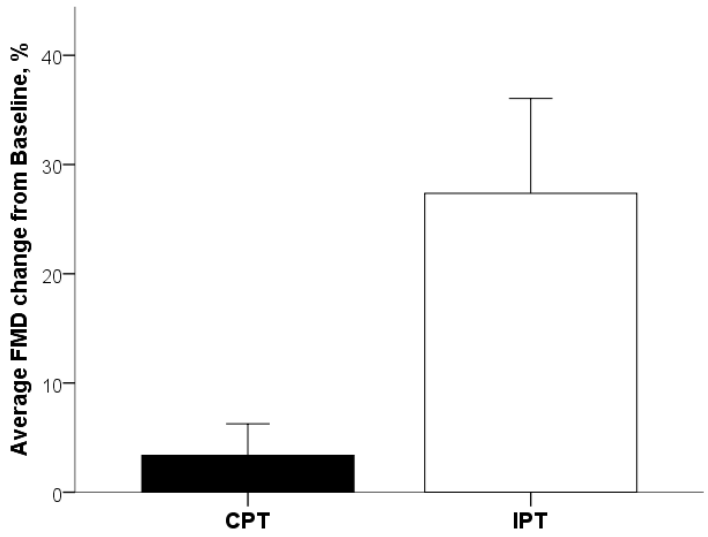


Figure 29 *Average FMD change from baseline*

4.3.6. Markers of inflammation and adhesion

Statistically significant interaction between treatment and time for plasma levels of tumor necrosis factor-α (p=0.03), interferon-γ (p=0.04) and soluble P-selectin (p=0.02) were noted. At 6 months, patients in the IPT group had lower circulating levels of TNF-α (average adjusted between groups difference of 0.76 pg/ml, 95% CI, 0.08-1.44, p=0.02), interferon-γ (average adjusted between groups difference of 1.38 pg/ml, 95% CI, 0.26-2.49, p=0.01) and P –Selectin (average adjusted between groups difference of 21.38 pg/ml, 95% CI, 6.79-35.97, p=0.01) compared to the CPT group (Table 23).

Table 23 Circulating biomarkers at baseline and 6 months after treatment

Variable	Group	Baseline	6 months
Interferon-γ, pg/ml*	CPT	1.1(2.4)	2.0 (1.1)
	IPT	0.9(1.9)	0.6 (1.4)††
TNF- α , pg/ml*	CPT	3.7 (1.8)	4.1 (5.8)
	IPT	4.0 (1.7)	3.7 (3.2)††
s-Eselectin, pg/ml	CPT	24.8±20.2	23.2±8.3
	IPT	25.8±11.0	18.1±13.3††
s-Pselectin, pg/ml	CPT	118.8±35.7	104.8±26.8
	IPT	103.1±30.1	82.1±22.5††

*Values are expressed as means±SD or *median (interquartile range) for non-normally distributed variables. †† P<0.05 compared to baseline*

No statistically significant between group difference at 6 months was detected for rest of the markers analysed (Table 24).

Table 24 Non significant between group differences a t 6 months

Variable	Unadjusted between group difference at 6M	95% CI	p value
CRP	1.15	-3.59- 5.89	0.627
IL-6	1.21	-0.65-3.06	0.196
IL-8	1.62	-8.32-11.56	0.744
IL-10	3.17	-1.38-7.71	0.167
IL-12	0.48	-0.23-1.2	0.182
E-Selectin	0.73	-5.44-6.9	0.810
ICAM-3	0.12	-0.08-0.32	0.233
Thrombomodulin	0.45	-0.31-1.21	0.241

4.3.7. Metabolic parameters

Patients in the IPT group exhibited an improved metabolic control 6 months after therapy compared to CPT patients (average between group difference of 0.65%, 95%CI 0.22-1.14, p=0.003). In addition a statistically significant reduction in Glucose (average difference between group 1.55mmol/l, 95%CI 0.25-2.85, p=0.012) was observed in IPT when compared to CPT patients.

4.4. DISCUSSION

This is the first study to show that mtROS production can be modulated in humans and that these changes are associated with a reduction of systemic inflammation, improved metabolic control and endothelial function in patients with T2DM. These results suggest mtROS may play an important role in the relationship between systemic inflammation and risk of CVD in T2DM and PD. Previous reports documented mitochondrial dysfunction and altered oxidative stress production in patients with PD and T2DM. However, we are the first to show that mtROS production can improve following intensive PD treatment. This was associated with a better control of systemic inflammation and a general improvement of PD. mtROS production is increased following activation of immune-inflammatory cells[639,640] and is considered a crucial step for pro-inflammatory cytokine production[262,263,641]. The lower mtROS observed after IPT may reflect a reduced activation of the immune-inflammatory system due to better oral health and reduced bacteraemia, while the lower circulating levels of IFN-γ may result from the reduced intracellular concentration of mtROS. In keeping with this hypothesis, we show that IPT is associated with a greater reduction of mtROS in lymphocytes, a subpopulation of PBMC that produces IFN-γ under condition of increased intracellular levels of oxidative stress. IFN-γ is an essential regulator of immune function. In viral or bacterial infection, IFN-γ is at the top of the inflammatory cascade as it is secreted by activated T cells and can subsequently activate macrophages, resulting in the secretion of other pro-inflammatory mediators. Therefore, our data suggests that a dysregulated production of mtROS may be a reversible alteration, which accounts for the immune-inflammatory deficits commonly described in people with PD and T2DM. We previously documented that IPT induces a significant improvement of endothelial function in patients with PD 6 months after

dental treatment in an otherwise healthy population affected by generalized severe PD[12]. However, the underlying biological pathways accounting for these results remained unclear. We now replicate this finding in subjects with T2DM and moderate to severe PD and show that changes in LPS serum profile, IFN-γ and mtROS production track changes of FMD. At least two mechanisms could account for these findings. Firstly, IFN-γ could act as a transducer between activated inflammatory cells and endothelial cells. IFN-γ is known to induce expression of endothelial cell adhesion molecules, such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, which are early markers of atherogenesis and are crucial for leukocyte recruitment to the plaque.[60] Their expression can be further intensified by cross talk between IFN-γ and lipopolysaccharide signaling.[61] Therefore, it is possible that a lower concentration of LPS detected in the IPT group could be related to a reduced bacteraemia achieved by better oral health combined with reduced circulating levels of IFN-γ following IPT and account for the improved endothelial function recorded at the end of the trial. Secondly, the measure of mtROS in circulating inflammatory cells could reflect a more generalised state of increased mtROS production in other vascular compartments. In patients with T2DM, the up-regulation of mtROS production in circulating leukocytes is associated with higher mtROS production in arterioles isolated from subcutaneous fat[642,643]. Furthermore, impaired endothelium-dependent vasodilation in freshly isolated arterioles from diabetic individuals is reversed by mild membrane depolarization or mitochondria-targeted antioxidants[642]. mtROS production in circulating leukocytes could have considerable potential as a reliable marker of mtROS in endothelial cells, with the advantage of a measure which can be made in readily available blood cells. Our study has several strengths but also some limitations. It is a randomised controlled trial with vascular and laboratory technicians blinded to

the patient treatment allocation. Our group was the first to describe, characterise and validate the impact of IPT on endothelial function, markers of inflammation and oxidative stress. We established and validated the use of FMD to measure endothelial function, optimising its reproducibility for large longitudinal studies and clinical trials. All these factors highlight the robustness of our results. In addition, FACS analysis does not represent the most accurate assay to measure mtROS. However, it should be recognised that the gold standard techniques used to measure mtROS are normally laborious and cannot be used in human clinical trials. The high specificity and sensitivity of MitoSOX for mitochondrial superoxide production has been repeatedly documented and the probe is currently considered the gold-standard method for mtROS assessment by FACS analysis. To minimise artefacts and optimise our assay, we carefully followed cell isolation and staining instructions reported on previously validated protocols and we acquired the FACS results immediately after staining. Furthermore, as staining with other fluorescent probes is known modify mitochondrial function and influence mtROS, we performed gating using the physical characteristics of the cell populations rather than fluorescent-labelled antibodies. While this precluded the opportunity to correlate mtROS with markers of lymphocytes/monocytes activation, it excluded an important source of variability to our results.

4.5. Conclusions

A reduced mtROS production following IPT is associated with a general improvement of inflammation, metabolic control and endothelial function in patients with T2DM. mtROS could represent an important and novel therapeutic target to reduce risk of cardiovascular disease and other inflammatory complications in patients with T2DM.

5. STUDY 3: Effects of periodontal treatment on vascular phenotypes in patients with type 2 diabetes. A 12 months randomized controlled clinical trial.

5.1. Introduction

Atherosclerosis is a systemic disease affecting large and medium-sized arteries. Atherosclerotic lesions are asymmetrical focal thickenings of the innermost layer of the intima, consisting of cells, connective-tissue elements, lipids and debris[644]. Atherosclerosis and its clinical manifestations, such as MI and stoke, are the main causes of death and disabling diseases in Europe, the United States and much of Asia[645,646]. At present, CVD cause almost 40 percent of all deaths in North America and are the most common cause of death in European men under 65 years, and the second most common cause in women[293]. Furthermore, due to a rapidly increasing prevalence of obesity and diabetes, CVD is expected within the coming years to be the leading cause of death globally. Diabetes is an established risk factor for atherosclerosis. The hyperglycemia can lead to the formation of advance glycation end products (AGEs)[317]. By binding surface receptors such as RAGE (receptor for AGEs), these AGE-modified proteins can trigger the release of pro-inflammatory cytokines and stimulate other inflammatory pathways in the vascular endothelium. In addition, the diabetic state promotes oxidative stress mediated by reactive oxygen species and carbonyl groups[647]. Inflammation may represent a link between diabetes to atherosclerosis. Infection and/or inflammation have been associated with the initiation, progression and complications of atherosclerosis[107], but it is uncertain if the link observed in humans is

causal. If it is, questions exist about the mediators and mechanisms involved, and how the response might be modified for therapeutic benefit. The assessment of the c-IMT is a noninvasive and reproducible imaging parameter to evaluate atherosclerosis. The thickness of the carotid arteries has been considered an early marker of atherosclerotic disease and a target for pharmacological treatment of atherosclerosis. B-mode ultrasound can directly assess the c-IMT correlating with histologic intima and media layers[523,542]. Observational evidence suggest that for each 0.03-mm increase per year in carotid arterial intima-media thickness, the relative risk for nonfatal myocardial infarction or coronary death was 2.2 (95% CI, 1.4 to 3.6) and the relative risk for any coronary event was 3.1 (CI, 2.1 to 4.5) ($P < 0.001$)[648]. B-mode ultrasound scan of c-IMT is also adopted in the screening of patients with CAD[649,650]. Increased c-IMT was associated with a significantly higher incidence of stroke and myocardial infarction[526]. The Cardiovascular Health Study Collaborative Research Group has shown in 4476 individuals without clinical cardiovascular disease followed up over 6 years, that the relative risk for myocardial infarction or stroke for the quintile with the highest IMT as compared with the lowest was 3.87[511]. In addition, atherosclerosis in coronary arteriography correlates with the presence of lesions in the carotid arteries[510]. Diabetes is a condition associated with high risk of CV complications and faster c-IMT progression[651]. Evidence from more than two decades suggested the association between PD, diabetes and CVD. In addition, our group, in a systematic review and meta-analysis, has reported an association between PD and c-IMT[7]. The nature of the association remains still unclear since data from observational studies do not allow determining causality. In absence of experimental studies on the impact of periodontal treatment on CV events such as stroke and myocardial infarction, the current research has focused on surrogate measures of CVD. Flow-mediated dilatation, as previously

discussed in this Thesis represents a non-invasive measure of endothelial function and predictor of future cardiovascular risk. FMD 6 months following periodontal therapy has been assessed in previous trials[12,413] and in Chapter 4 of this dissertation. However, FMD has been measured 6 months after therapy therefore designing trials with a longer follow-up could add more information on this association. Piconi et al. have evaluated changes in c-IMT after periodontal treatment in a trial with no control group and not adhering to the guidelines for the measurements of c-IMT diminishing the importance of their finding[399]. Kappelas et al. have conducted a RCT analyzing changes in c-IMT 12 months following a single session of periodontal therapy in a population represented by Aboriginal Australians and reporting a reduction in the carotid intima-media thickness[652]. The systematic review and meta-analysis of observational studies have presented in Chapter 3 suggest an association between PD, c-IMT and FMD and propose the conduction of experimental trials investigating the causal features of this association[7]. Our group has designed a randomized, controlled trial to determine the effect of a periodontal intervention on the progression of carotid intima-media thickness, an established noninvasive measure of subclinical atherosclerosis in patients affected by PD and T2DM.

5.2. Methods

5.2.1. Study Design

Eligible participants were consecutively enrolled into the study if they had: T2DM for ≥ 6 months diagnosis, moderate to severe PD (≥ 20 periodontal pockets with probing pocket depths >4 mm and marginal alveolar bone loss >30%) and a minimum of 15 teeth. Exclusion criteria included: i) uncontrolled systemic diseases other than diabetes ii) Hepatitis B or HIV infection, iii) chronic treatment with medications known to affect

periodontal tissues, iv) chronic systemic antibiotic treatment, v) pregnancy or lactation. All patients gave written informed consent, and the study was approved by the local ethics committee (Ref 07/H0714/97).

5.2.2. Periodontal Examination

Clinical periodontal data were recorded at baseline and 2, 6 and 12 months after the therapy as described in Chapter 2. This data included PPD and gingival recession at six sites per tooth. The presence or absence of supra-gingival dental plaque and gingival bleeding on probing was also recorded.

5.2.3. Periodontal Therapy

Essential dental care including removal of compromised teeth was provided within the trial. Oral hygiene instructions were given to all study participants. Patients in the IPT group received a single session of full mouth scaling and root planing under local anesthesia. At 2 months, those patients with good oral hygiene (full mouth dental plaque scores < 20%) and ≥ 6mm residual periodontal pocket underwent periodontal surgical corrective therapy (20 participants). Patients who presented with suboptimal oral hygiene received corrective treatment in form of additional scaling of the root surfaces under local anesthesia every 3 months until completion of the study. CPT patients received supra-gingival scaling and polishing at baseline, 2, 6 and 9 months. At the end of the study, all CPT patients received any required additional periodontal therapy. Any patients showing progression of PD[522] during the trial were withdrawn from the study and treated accordingly.

5.2.4. Inflammatory, vascular and metabolic assays

Blood samples were taken at baseline, 6 and 12 months follw-up. Serum C-Reactive Protein (CRP) concentrations were determined by immunoturbidimetry (Cobas Integra

700, Roche, Mannheim, Germany). Full blood differential count was assessed by standard biochemistry whilst Interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, Interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α and endothelial cell surface markers (E-selectin, P-selectin, Intercellular Adhesion Molecule (ICAM)-3 and Thrombomodulin) were measured by multiplex assay (Meso Scale Discovery, Maryland, USA). Inter- and intra-assays coefficient of variation were <7%. HbA1c (liquid chromatography) was measured in plasma on an automated analyzer (Cobas 8000 analyzer, Roche Diagnostics Corp).

5.2.5. Vascular outcomes

5.2.5.1. c-IMT

c-IMT was assessed at baseline, 6 and 12 months after periodontal therapy. The right and left common and internal carotid arteries and the radial artery were scanned with a linear-array transducer (Acuson XP10 7-MHz, Visualsonic 55-MHz) as previously described in the methods. IMT was calculated as the distance between the first bright line (lumen-intima interface) and the leading edge of the second bright line (media-adventitia interface). Six measurements, the 3 maximum measurements of the right common carotid artery in 3 different frames and the 3 maximum measurements of the left common carotid artery in 3 different frames were averaged.

5.2.5.2. Flow-mediated dilatation

FMD was measured at baseline, 6 and 12 months after periodontal therapy. Endothelium-dependent and -independent FMD was assessed by ultrasound imaging of the brachial artery with a high-resolution probe (7-MHz), as described in Chapter 2.

5.2.6. Outcomes Assessment

The primary outcome was the between group difference in c-IMT at 12 months. Secondary outcomes included differences in: i) HbA1c at 12 months, ii) FMD at 12 months, iii) periodontal clinical parameters, inflammatory and endothelial circulating markers at 12 months.

5.2.7. Statistical Analysis

A minimum sample size of 52 participants per group was required to detect a 0.02mm difference in c-IMT at 12 months between treatment groups, with a standard deviation of 0.036 (derived from published reports[399,652]), α=0.05 and 80% power (assuming a 10% loss to follow up rate, a total of 58 patients per group were considered). Data are reported as mean and standard deviation unless otherwise specified. All analyses used the intention-to-treat population with last measure carried forward approach for missing values. Primary outcome was analyzed with analysis of covariance (including baseline values as independent variable) at 12 months. Covariates included also age, gender, body mass index, smoking and treatment group. Repeated ANOVA models were also created to investigate changes in primary and secondary outcomes over time between study groups. A conservative F-test was used to interpret the model using the Greenhouse-Geisser correction to account for compound symmetry violations. Univariate analyses were performed with crude values of each outcome variable between study groups at different time points. Further multivariate models were constructed including variables that showed a statistically significant association with the outcome from univariate analyses and they included age, gender, smoking, body weight and ethnicity.

A two-sided value of $p < 0.05$ was considered statistically significant. SPSS version 22 and STATA version 12 were used.

5.3. Results

5.3.1. Patients characteristics

From October 2010 to October 2012, 270 patients with T2DM were screened (Figure 30) and 117 eligible patients were randomized to either IPT (N=59) or CPT (N=58). Enrolled patients were predominantly middle-aged and equally distributed in gender of white background and smoking habits (Table 25). 3 participants in the IPT and 5 in the CPT group were lost to follow-up. Adverse events were comparable between study groups as well as changes in prescribed medications.

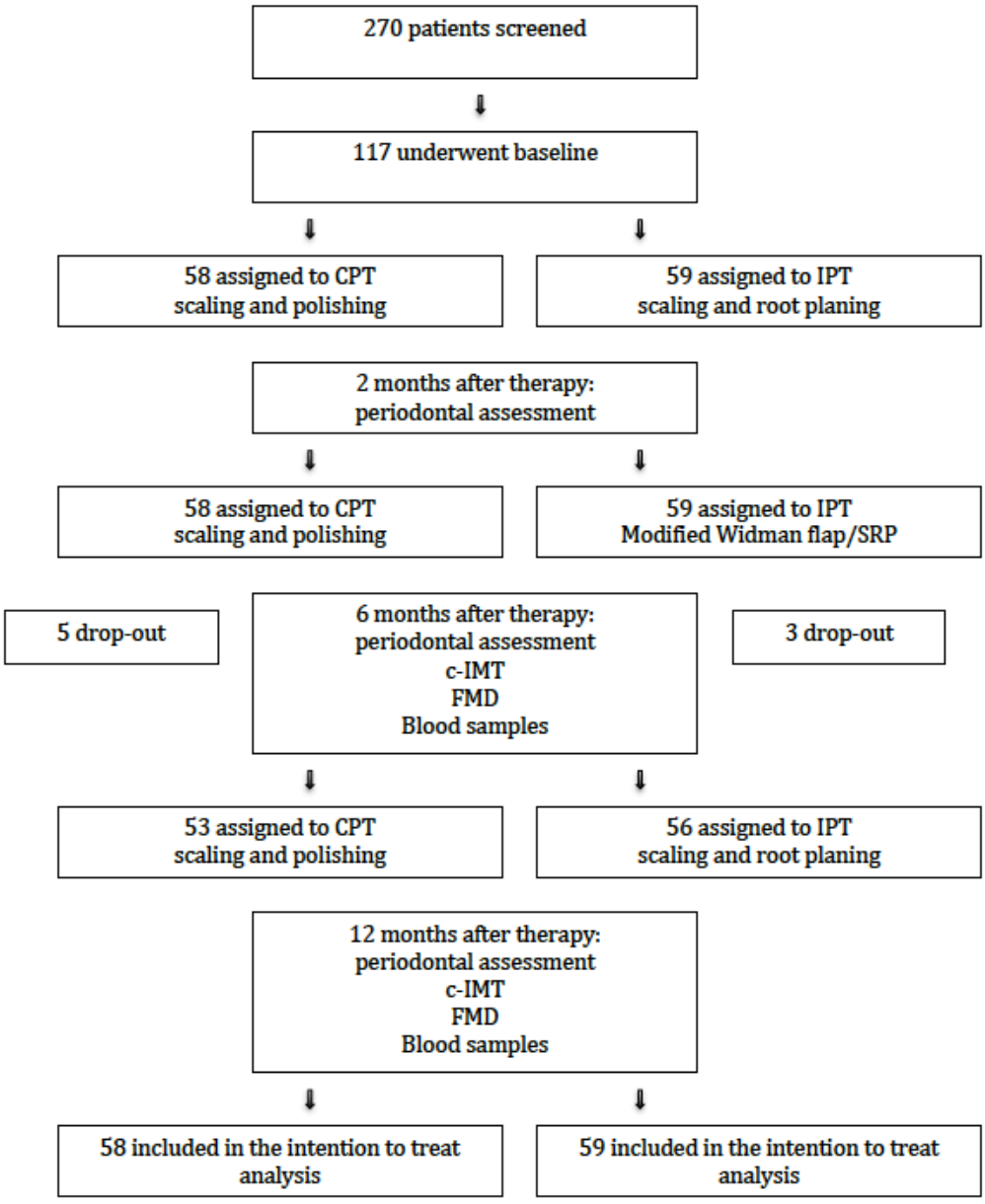


Figure 30 *Study Flowchart*

Table 25 Baseline characteristics of the study participants

Variable (mean±SD)	CPT (N=58)	IPT (M=59)
Age, years	59±10	56±12
Gender, Males	35(59%)	32(62%)
Ethnicity, Caucasian	24(41%)	17(29%)
BMI, Kg/m^2	30±5	30±6
Smoking, Current	9(15%)	9(15%)
Systolic BP, mmHg	138±17	145±16
Diastolic BP, mmHg	83±10	84±10
HbA1c (%)	8±1.8	7.9±1.7
Cholesterol, mmol/l	4.2±1.1	4.3±1.2
HDL, mmol/l	1.2±0.4	1.3±0.4
LDL, mmol/l	2.3±0.9	2.3±1
FMD%	4±2	4±3
c-IMT mm	0.64±0.06	0.61±0.08
CRP*, mg/l	1.8 (3.1)	1.7 (3.0)

*Values are expressed as means±SD or *median (interquartile range) for non-normally distributed variables.*

5.3.2. Periodontal clinical outcomes

At the end of the trial, all clinical periodontal parameters were significantly better in IPT patients compared to CPT (Table 26). Dental plaque and gingival bleeding scores were lower in the IPT group at 12 months compared to CPT. Probing pocket depths were reduced 12 months after therapy in IPT compared to CPT patients

Table 26 Periodontal parameters at Baseline and 6 Months after Periodontal Therapy

Variable	Group	Baseline	12 months
FMPS, % §	IPT	78±16	38±18
	CPT	76±16	58±23
FMBS, % ¶	IPT	66±18	29±16
	CPT	66±17	54±22
PPD, mm	IPT	4±0.8	2.8±0.6
	CPT	3.9±0.8	3.6±1
REC, mm	IPT	1.1±0.8	1.7±1
	CPT	1.3±0.8	1.6±0.9
NPKTs, n *	IPT	54±25	11±13
	CPT	52±24	38±27

Values are expressed as means±SD.
† P<0.001 for the comparison with the standard-treatment group.
IPT: intensive treatment group, CPT: control treatment group, FMPS: full mouth plaque score, FMBS: full mouth bleeding score, PPD: periodontal pocket depth, REC: gingival recession, NPKTS, number of pockets.
¶ Scores for full-mouth gingival bleeding were calculated for each patient as the number of sites with gingival bleeding on probing divided by the total number of sites per mouth, multiplied by 100.
*§ Scores for full-mouth plaque were calculated for each patient as the number of sites with detectable plaque divided by the total number of sites per mouth, multiplied by 100.**
** Periodontal pockets identified as sites with probing greater or equal to 5 mm*

5.3.3. Vascular outcomes

5.3.3.1. c-IMT

c-IMT progression was significantly lower in IPT patients compared to CPT at 12 months after therapy (Figure 31). The average between groups difference in c-IMT was 0.025mm (95% CI, 0.003-0.048, p<0.05) at 12 months.

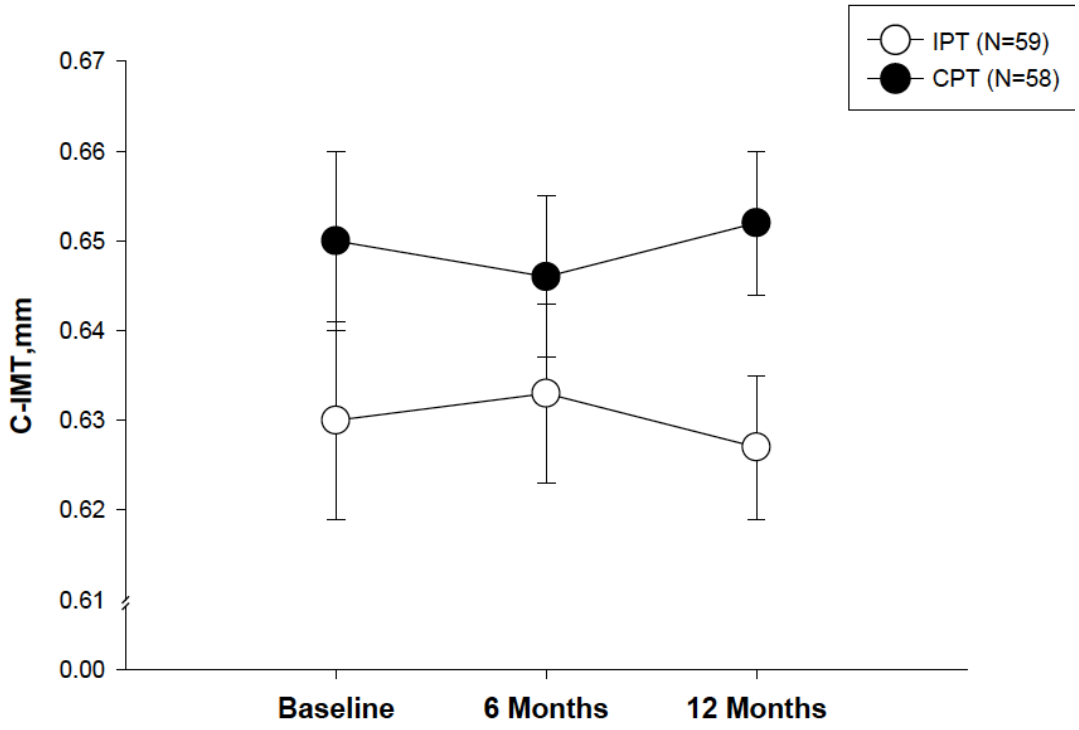


Figure 31 *Changes in c-IMT during the study period*

5.3.3.2. FMD

FMD was greater in IPT patients compared to CPT at 12 months after therapy ($p<0.05$) (Figure 32). The average between groups difference in FMD was 1.05 % (95% CI, 1.36-2.47, p=0.002) at 12 months.

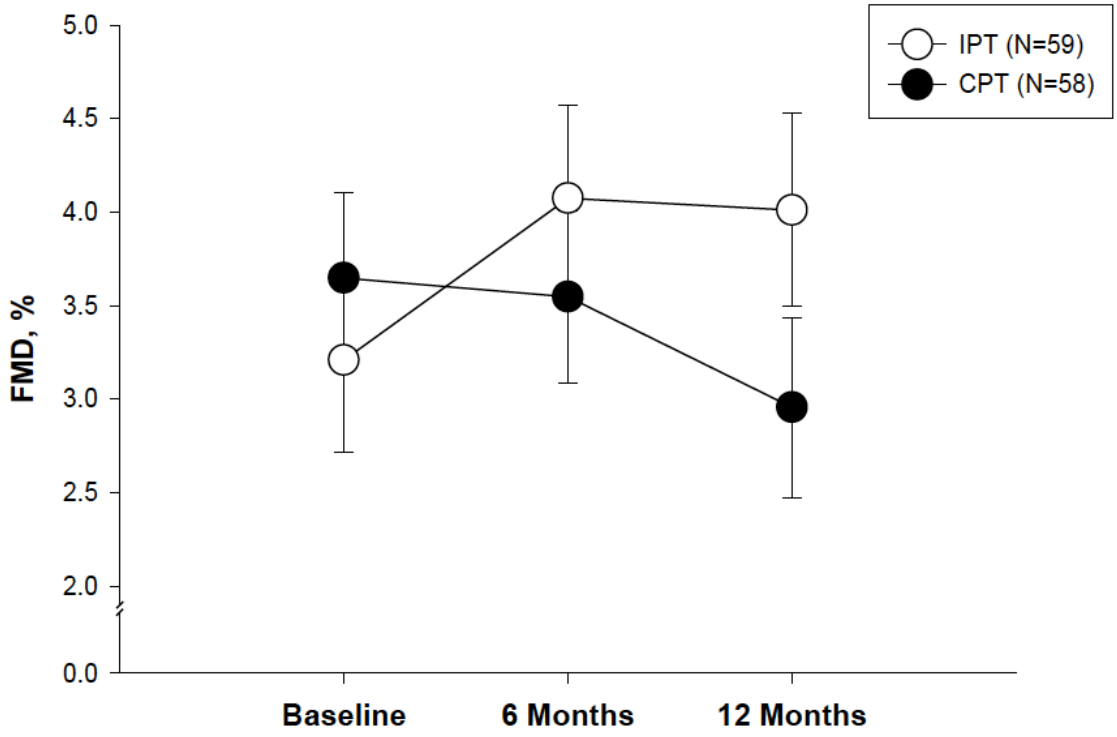


Figure 32 *Changes in FMD during the study period*

No interaction between treatment and time was observed for nitroglycerin-dependent dilatation or for brachial artery diameters and no changes of the nitroglycerin-dependent dilatation were observed from baseline to 12 months. (unadjusted difference of 0.5; 95% CI, -0.8-3.8; p=0.75).

5.3.4. Markers of inflammation and vascular activation/damage

IL-10 serum levels were lower in the CPT compared to the IPT group (Figure 33). The average between groups difference in IL-10 levels was 4.73 pg/ml (95% CI, 2.73-6.73, p=0.002) at 12 months. No substantial differences in other biomarkers were noted (Table 27).

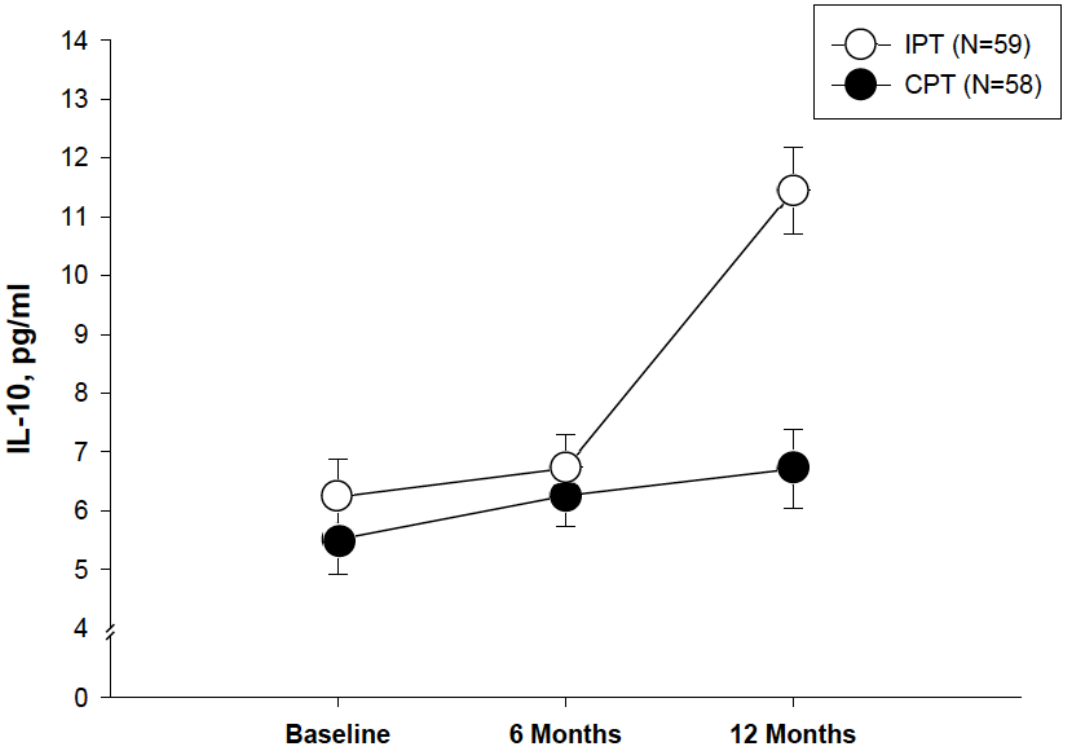


Figure 33 *Changes in IL-10 during the study period*

Table 27 *Non-significant between group differences a t 12 months*

Variable	Unadjusted between group difference at 12M	95% CI	p value
IL-6	-1.71	-5.46-2.05	0.37
IL-8	8.26	-1.53-18.05	0.10
IL-12	21.42	-31.87-74.71	0.42
TNF-α	0.002	-0.97-0.98	1.00
IFN-γ	-0.14	-3.14-2.86	0.92
E selectin	2.64	-14.26-19.53	0.76
P selectin	7.57	-28.16-43.29	0.67
Thrombomodulin	0.86	-5.60-7.32	0.79
ICAM 3	1.31	-2.29-4.90	0.47

5.3.5. Metabolic parameters

Metabolic control improved in IPT patients after 12 months treatment compared to CPT. The average between groups difference in HbA1c levels was 0.95 % (95% CI, 1.84-0.06, p=0.002) at 12 months (Figure 34).

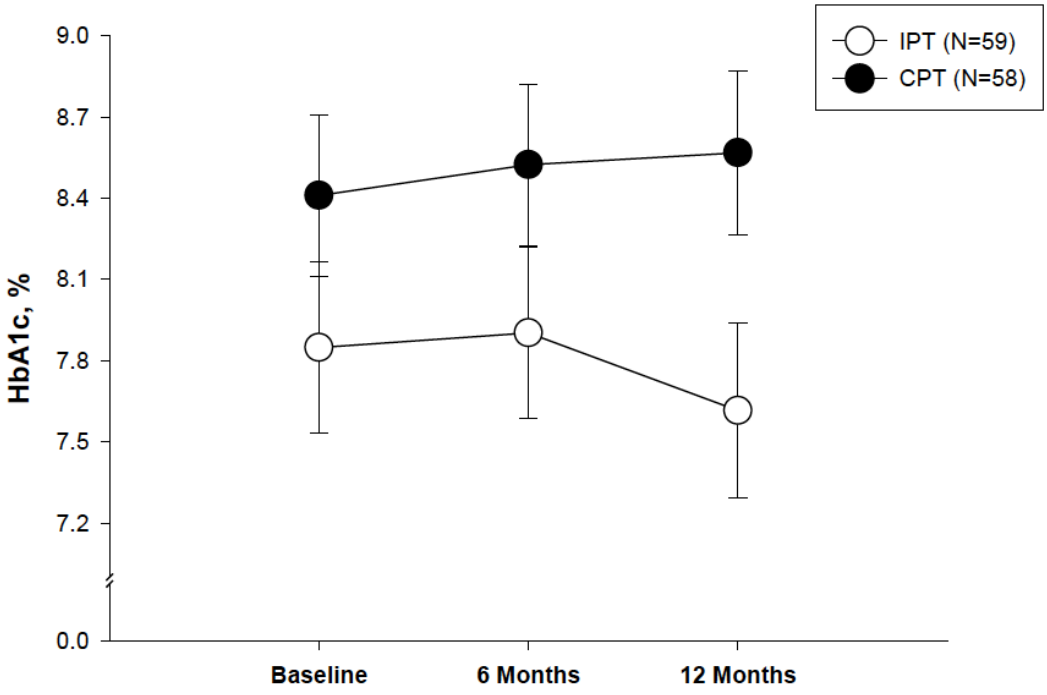


Figure 34 *Changes in HbA1c during the study period*

5.4. Discussion

In this trial, we have observed a statistically significant difference in the progression of c-IMT 12 months after periodontal treatment in patients affected by moderate to severe PD and T2DM. This effect was associated with an improvement of the endothelial function, metabolic control and IL-10 profile. The control of the periodontal infection seems to have favorable effect on the human vasculature. In chapter 4 we have also described a lower circulating level of LPS suggesting a systemic benefit of the periodontal therapy. Circulating bacterial endotoxin is well established to provoke severe endothelial dysfunction and exhibits a variety of pro-atherogenic features[144,653-655]. Endotoxemia does not occur only in sepsis but also occurs in otherwise healthy individuals [656]. Chronic or recurrent bacterial infections may represent sources of circulating bacterial byproducts[657]. The hypothesis that infections could contribute to the pathogenesis of atherosclerosis has been extensively researched over the last 20 years[658-661]. Seroepidemiologic studies supported an involvement of common chronic infections in atherogenesis[658,661]. Specifically, herpes viridae, gram-negative bacteria (Helicobacter pylori , Chlamydia pneumoniae) and chronic dental infections were associated with atherosclerosis[142,144,658,662,663]. However, there is no evidence reporting that infections are causally related to atherosclerotic disease [664]. Common chronic infections, including PD, have been suggested as markers of increased risk for carotid arteries atherosclerosis in a prospective population-based survey [343]. Chronic infections such as PD, could contribute to CVD even in individuals in the low-risk category and in absence of common traditional vascular risk factors such as smoking, obesity, hypertension and dyslipidemia. Approximately 10^8 – 10^{12} bacteria can be detected in periodontal lesions and it has been reported that a large number of oral microorganisms, including those associated with PD, can enter the circulation

through the microvasculature following tooth brushing and other dental invasive procedures[665]. Indeed, using PCR techniques, bacteremia was confirmed in blood samples taken from 30 patients after ultrasonic scaling, periodontal probing and tooth brushing [666]. Further, data from an observational study including 194 patients with PD, suggested that periodontal site bleeding after tooth brushing was associated with approximately 8-fold increase in bacteremia[667]. Similarly, bleeding on probing has been associated with systemic inflammation and bacteremia[463,667]. Periodontal bacteria could reach the vascular endothelium through two mechanisms; either direct invasion as a consequence of bacteremia or dissemination via internalization in migrating phagocytic cells. P. gingivalis and A. actinomycetemcomitans have both demonstrated migration capacities[668,669] . Eikenella corrodens and Prevotella intermedia were also shown to invade human endothelium and smooth muscle cells[670]. DNA from periodontal organisms such as A. actinomycetemcomitans and P. gingivalis was detected in atherosclerotic lesions by PCR. Using 16S rDNA PCR, it was reported that 1.5-2.2% of the total DNA in the atheroma samples was bacterial with a large proportion of oral origin. P. gingivalis had the higher incidence[671]

IL-10 is a potent anti-inflammatory cytokine[672-674] secreted primarily by the macrophages and Th2 subtype T lymphocytes. Evidence form an animal model report that it preserves endothelial function after an inflammatory stimulus[675] and impedes mechanisms of endothelial dysfunction in diabetes[676]. IL-10 protects endothelial function after LPS treatment by attenuating increases in superoxide in the vessel wall[675]. In addition, it inhibits the synthesis of pro-inflammatory cytokines by activated monocytes and reduces the adhesiveness of monocytes to stimulated endothelial cells in vitro inhibiting endothelial cell adhesion molecules ICAM-1 and VCAM-1[677,678]. Furthermore, IL-10 knock-out mice develop severe atherosclerosis in response to

hypercholesterolemia[679]. IL-10 protective features include inhibition of macrophage activation, matrix metalloproteinase, pro-inflammatory cytokines and cyclooxygenase-2 expression in lipid-loaded and activated macrophage foam cells. Recent evidence also reports its ability to alter lipid metabolism in macrophages[679]. Periodontal treatment seems to be effective in elevating systemic IL-10 levels in patients with T2DM[680]. In addition a recent meta-analysis reported a favorable effect of periodontal treatment on serum inflammatory levels such as TNF-α and CRP of subjects with T2DM and PD[681]. We have previously reported the beneficial effect of periodontal therapy on FMD in T2DM at 6 months follow-up. This trial provides the evidence of a prolonged effect observable 12 months following treatment supporting the importance of controlling the periodontal infection in T2DM and its potential association with the pathogenesis of atherosclerotic disease. According to the response-to-injury model of atherosclerosis[502], various factors can cause alterations in the endothelium predisposing arteries to the development of atherosclerosis. Changes in the adhesiveness of the endothelium to leukocytes, its permeability, and expression of vasoactive molecules could favor atherogenesis[275]. Increased c-IMT in subjects with impaired FMD is associated with CV risk factors[682] supporting the concept that endothelial function might reflect the propensity of arteries to develop atherosclerosis[683]. Impaired brachial FMD has been related to increased carotid IMT[682] suggesting an inverse relation between brachial FMD response and c-IMT[684,685]. Plausible pathophysiological mechanisms linking infections and atherosclerosis could include direct infection of the arterial wall[664,686], inference of acute-phase response with a pro-coagulant state and induction of autoimmunity[687-690]. In addition, endotoxemia can cause endothelial dysfunction in the course of severe systemic infections, thus driving vasculature into a pro-atherogenic mode[664]. LPS from

Escherichia coli augments the expression of endothelial adhesion molecules and subsequent leukocyte adherence[691-693] and is considered an activator of cytokine production in atheroma[655]. Further atherogenic properties include induction of intravascular coagulation and lowering of HDL, which is a natural scavenger of endotoxins[694]. Exposure of human volunteers to LPS provokes endothelium dysfunction in vivo[296]. Subclinical atherosclerosis should preferably detected at an early stage allowing primary preventive measures[695]. The development of atherosclerosis usually takes decades, and the thickening of the arterial wall is one of the first detectable signs. B-mode ultrasound measurement of c-IMT is frequently used for non-invasive evaluation of subjects at risk of atherosclerosis. Still, the exact risk of cardiovascular events associated with an increased c-IMT in general populations is not entirely clear. Evidence from the Carotid Atherosclerosis Progression Study (CAPS), reported that the incremental predictive value of c-IMT in the general population might not necessarily improve the CV classification. C-IMT measured by B-mode ultrasound is associated with future CV events, however when a model using the classical risk factors was compared with a model including c-IMT, the classification of individual risk did not result consistently improved[607]. On the contrary, the maximum IMT value along the whole carotid artery or the presence of plaques seem to have the better prognostic value[696]. The improved glycemic control observed in our population 12 months following the periodontal therapy needs validation with a sample size adequate to answer such research question. However, a reduced level of Hb1Ac would support a lower vascular risk expressed by an amelioration of brachial FMD, c-IMT and IL-10 profile. In our study we have adopted a standardized protocol to assess both FMD and c-IMT. In addition, the same vascular technician has acquired and analyzed all the vascular data reducing potential sources of variability. We have demonstrated

that treating PD could improve c-IMT. However, thickening of the carotid intima-media layers could not necessarily represent subclinical atherosclerosis. The presence of atherosclerotic plaque on carotid B-mode ultrasound is a better individual predictor of future cardiovascular events than c-IMT. Therefore, our results support the association between PD and c-IMT but do not prove that periodontal treatment can effectively lower the future CV risk.

5.5. Conclusions

The c-IMT reduction 12 months following periodontal therapy supports a link between PD and atherosclerosis. Further studies should evaluate the mechanism by which periodontal treatment could reduce the c-IMT progression. To evaluate the impact of periodontal health on CV conditions, large scale/multinational intervention trials should be conducted on the effect of periodontal therapy on hard CVD endpoints such as myocardial infarction and stroke identifying settings representing the every-day periodontal clinical care.

6. STUDY 4: Remote ischemic preconditioning (RIPC) reduces endothelial dysfunction in a human model of systemic inflammation

6.1. Introduction

The phenomenon of ischemic preconditioning was firstly described in a canine experimental model in 1986[697]. Murry et al. reported a substantial reduction in the extent of a myocardial infarction area exposing the circumflex coronary artery territory to brief periods of ischemia as four cycles of 5 min of ischemia followed by reperfusion before 40 minutes of complete occlusion of the artery. This finding suggested that the myocardial tissue could be prepared prior to an ischemia-reperfusion insult reducing the subsequent damage extent. However, a longer period of occlusion, 3hours, nullified the protective effect of this procedure. The myocardium is not the only tissue responsive to the ischemic preconditioning, numerous studies have proven similar effects in other body tissues. The mechanisms involved in ischemia preconditioning are multiple and not fully elucidated. The induction of a cascade of intracellular kinases and changes in the mitochondrial function, via opening of ATP-sensitive potassium channels and closure of mitochondrial permeability transition pores have been suggested[698,699]. However, their role and nature needs to be fully understood[700,701]. The window of protection observed after the preconditioning procedure has also been extensively researched. Its vascular protection vanishes after few hours, however Marber et al. described a secondary effect starting in the following 24-48 hours, defined as a second window of protection, persisting for up to 3-4 days[702].

Subsequently, it was reported that a brief ischemia of a coronary artery could protect the myocardial territory not perfused by the same vessel, introducing the concept of intra-organ preconditioning[703]. Further research then reported myocardial protection subsequent to transient ischemia of the kidney or the small bowel[704,705] suggesting the concept of remote ischemic preconditioning (RIPC). This theory has been supported by the multi-organ protection obtained following transient ischemia of a wide range of tissues[706,707]. RIPC, compared to local preconditioning, shares similar mechanisms providing two phases of protection; recent evidence however suggested differences in the pathways underlying the two procedures[708]. Kharbanda et al. described the protective action of ischemic preconditioning on the endothelial dysfunction following ischemia reperfusion damage in a human model[709]. Furthermore, Loukogeorgakis et al. reported that RIPC prevents endothelial dysfunction following ischemia reperfusion injury in conduit vessels identifying two temporally distinct phases of protection. A first window presented straight after RIPC and lasting approximately 4 hours followed by a second phase detectable 24 hours after the RIPC stimulus and continuing for at least 48 h[577]. In addition, the authors demonstrated that RIPC exert its protective effects via an intact autonomic function. The endothelial function is involved in the regulation of multiple physiological processes such as the vascular tone, permeability, response to inflammation, wound healing and coagulation. Multiple stimuli including diabetes, hypercholesterolemia, oxidative stress, inflammation may induce endothelial dysfunction and lead to atherosclerosis. A decreased bioavailability of NO seems to be mechanism related to the alteration of the endothelial regulation of the vascular tone. Up regulation of the endothelial expression of adhesion molecules, immune cell recruitment to vascular wall, smooth muscle cell activation are all involved in the endothelial dysfunction[710]. Impaired NO-dependent vasodilatation has been related to

atherosclerosis[711]. Therefore, preventing endothelial dysfunction is of great importance in avoiding or ameliorating the development of CVD. Acute vascular inflammation is characterized by vasodilation, increased permeability of microvasculature, and vascular stasis. Endothelial cells undergo cytoskeletal changes that disrupt junctions 4–6 h after the mediator stimulus and lasting for days. The release of pro-inflammatory mediators such as IL-1, TNF-α, INF-γ mainly by lymphocytes and macrophages after stimulation by toxins, injury or inflammatory mediators leads to endothelial inflammatory activation, increased expression of selections, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) promoting the adherence of leukocytes[712]. Infections can provoke endothelial dysfunction. Hingorani et al. suggested that intramuscular injection of capsular polysaccharide typhoid vaccine produced a mild systemic inflammatory response in healthy volunteers that was associated with a transient impairment of FMD in the forearm circulation[296]. Periodontal treatment relies upon the mechanical cleaning of the diseased dentition, which reduces local bacterial load and clinical signs of gingival inflammation[713]. The treatment procedure often though results in an intense transient bacteremia as well as significant local gingival soft tissue damage[714]. Acute release of cytokines in serum (i.e. TNF-α) has been reported to occur as early as 60 minutes following dental cleaning. We have undertaken extensive characterization of the time-course and validation of a non-drug in vivo model to study human inflammation. A single intensive session of periodontal treatment (IPT) is associated with a one-week acute inflammatory response[11]. The bacterial and inflammatory burden produced during dental instrumentation also alters markers of coagulant pathways and markers of endothelial cell activation assessed by increased plasma concentrations of fibrinogen and D-dimers and soluble E-Selectin and Von Willebrand

factor respectively. The acute inflammatory response and haemostatic alterations correlate with the degree of the pre-existing periodontal infection (number of gingival pockets) and the extent of periodontal treatment (minutes of dental cleaning). The substantial inflammatory response generated by IPT, which is greater than that seen following vaccination, is also associated with profound and transient reductions in systemic endothelial function assessed by FMD of the brachial artery at 24 hrs. Therefore IPT might represent a valid model for studying inflammatory endothelial dysfunction since the inflammatory response generated is substantial (mean increase in serum CRP of 15-20 mg/L 24hrs after therapy), In addition, the effects on inflammatory cytokines, acute phase reactants, circulating markers of endothelial damage, coagulant factors and endothelial function itself are highly consistent. Furthermore, the duration of response which is sustained for up to one week from the therapy allows better assessment of the time course of the inflammatory changes in relation to the change in endothelial function; this could be important in identifying which components of the inflammatory response are most important in generating endothelial dysfunction. It is also a standard, safe therapy with benefits for the participant, since the procedure is associated with better periodontal health outcomes. IPT also allows the conduction of mechanistic studies on a larger scale than has hitherto been possible with other models since a large number of patients require treatment for severe periodontal disease. In animal models, an initial observation of endothelial dysfunction can be followed up by mechanistic experiments in vitro and in knockout models to dissect the likely cause(s) of the effect; deeper exploration in clinical studies is considerably more difficult. Since intensive treatment of periodontal disease induces an acute inflammatory response that transiently impairs endothelial function, we now wish to determine whether remote ischaemic pre-conditioning will

prevent acute inflammatory endothelial dysfunction observed 24 hours following IPT. This study would provide the first insight into whether the acute adverse vascular changes associated with inflammation can be modified by treatment. If so these findings would be of relevance to a range of clinical situations in which systemic inflammation has been associated with a short-term increase in risk of cardiovascular events.

6.2. Methods

6.2.1. Study Design

We designed a single blind, parallel group, randomized controlled trial to evaluate the effect of RIPC on endothelial function assessed by FMD within 7 days of IPT (Figure 35).

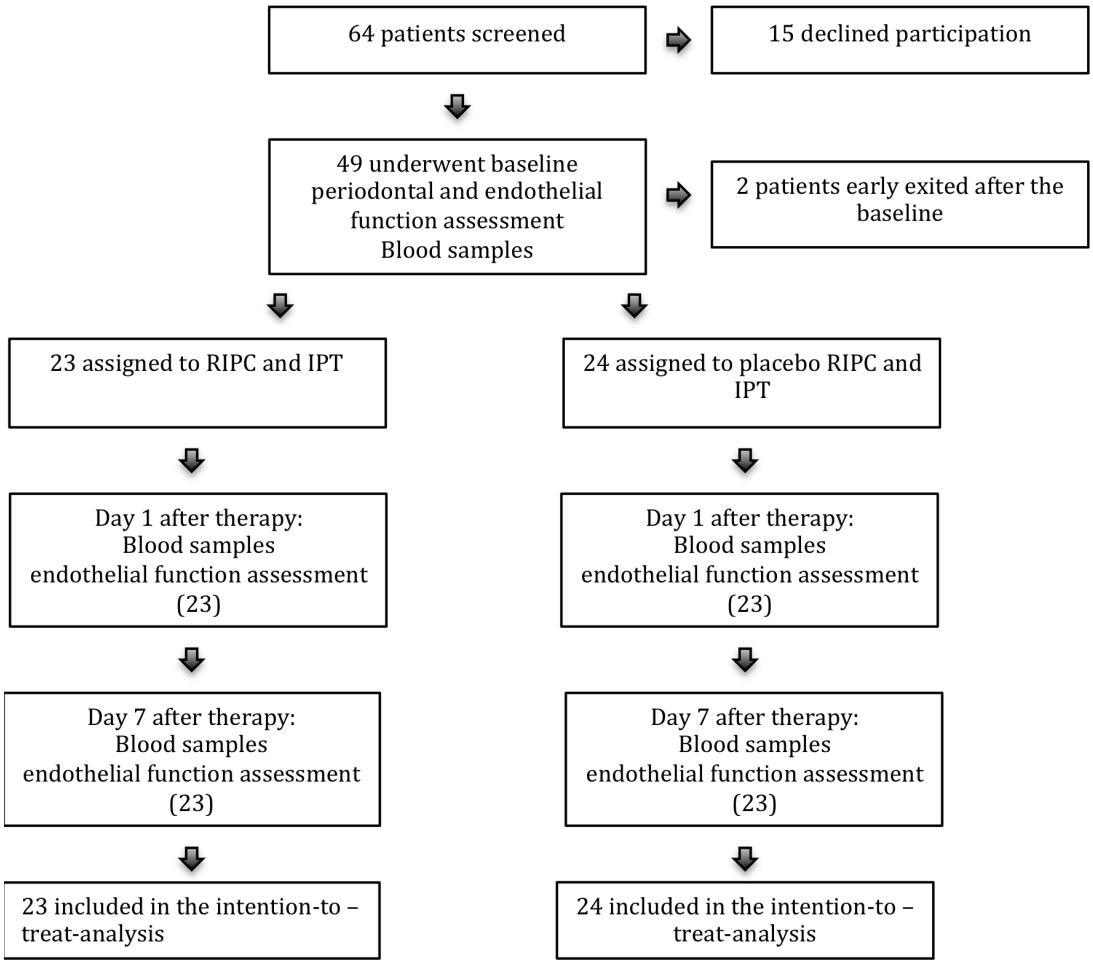


Figure 35 *Study Flowchart*

Consecutive patients referred to the Eastman Dental Hospital in London for periodontal therapy were invited to participate in this study if they had at least 30 periodontal pockets with probing pocket depth > 4mm and confirmed radiographic alveolar bone loss. Patient were excluded if they were: a) pregnant, lactating or of childbearing potential, b) on chronic treatment (i.e., two weeks or more) with specific medications known to affect periodontal status (phenytoin or cyclosporine) within one month of baseline visit, c) suffering from any systemic disease (assessed by medical history questionnaire), d) with limited mental capacity or language skills such that simple instructions cannot be followed or information regarding adverse events could not be provided, e) on any chronic medications or requiring antibiotic coverage for dental/periodontal procedures and f) had received a course of periodontal therapy in the preceding 6 months. All patients gave written informed consent. The study was approved by the London Queen Square Ethics Committee (06/Q0512/107).

A baseline periodontal examination was performed, and full medical and dental histories were collected by a single trained examiner. Arterial blood pressure was measured in triplicate, and the average of the readings was recorded. The study participants were randomly assigned with the use of a computer-generated table to receive intensive periodontal treatment preceded by RIPC (test group) or placebo RIPC (control) in a 1:1 ratio. To prevent an imbalance between the two groups with respect to smoking status, sex, age, and severity of periodontitis, restricted randomization (minimization) was performed by the study registrar[715]. Treatment assignments were concealed in opaque envelopes and revealed to the research staff performing the RIPC on the day the treatment was administered. Patients underwent dental examinations, blood samples and endothelial function assessment at Baseline, 1 and 7 days following periodontal treatment.

6.2.2. Periodontal examination and therapy

A single trained dental examiner at baseline recorded periodontal data. The data included, number of teeth, periodontal pocket depth (PPD) in mm, gingival recession and clinical attachment levels as previously described. The presence or absence of supragingival dental plaque and gingival bleeding on probing on whole mouth was also recorded. All study participants received standard oral hygiene instructions and underwent a single-sitting full-mouth session of scaling and root planing (IPT) by means of hand and ultrasonic instruments under local anesthesia within one month from the baseline. A single experienced clinician completed all treatments in a blind fashion (unaware of to the preconditioning assignment).

6.2.3. Remote ischemic preconditioning (RIPC)

RIPC consisted of three 5 minutes cycles of upper arm ischemia alternated by 5 minutes of reperfusion using a 9cm blood pressure cuff inflated to a pressure of 200mmHg. Sham procedure consisted of three 5 minutes cycles alternated by 5 minutes of reperfusion with the blood pressure cuff being placed around the upper arm with 10mmHg. IPT commenced after the completion of the RIPC protocol.

6.2.4. Vascular function

For this study, endothelium-dependent vasodilatation of the brachial artery was assessed using high-resolution ultrasound imaging (Acuson XP128 with a 7-MHz linear probe) in a quiet, temperature controlled room. All patients were fasting for a minimum of 6 hours prior to their assessment. Images of the right brachial artery were measured for 1 minute at baseline, 5 minutes of ischemia (using a blood pressure cuff inflated to 300mmHg) and during deflation. Flow measurements were derived by using a pulsed wave Doppler signal. Vessel diameter measurements were analyzed using

automated brachial software (Medical Imaging Applications, vascular research tools, version 5.6.7) and dilation was calculated as a percentage change from BL to the peak diameter. Endothelium-independent vasodilatation was also assessed following a 25µg dose of GTN administered sublingually. A single examiner who was blinded to the RIPC or sham procedure assessed all patients.

6.2.5. Inflammatory and vascular biomarkers

Serial blood samples were collected in fasting condition at baseline, 24 hours and 7 days after IPT. Samples were immediately processed and aliquots of serum and EDTA plasma were stored at -70 degrees as previously reported. At the end of the study all samples were processed in a blind fashion for a broad panel of inflammatory biomarkers using multiplex high sensitivity assays including interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) according to manufacturer's instructions (Meso Scale Discovery, Maryland, USA). Serum C-reactive protein (CRP) was measured by immunoturbidometry (Cobas, Roche Diagnostic, Mannheim, Germany). E-selectin, P-selectin, intercellular adhesion molecule-3 (ICAM-3) and thrombomodulin were assayed with a multiplex assay (Meso Scale Discovery, Maryland, USA)

6.2.6. LPS assay

A quantitative, endpoint assay for the detection of Gram-negative bacterial endotoxin was used (QCL-1000™, Lonza) was adopted. At the end of the study all samples were processed in a blind fashion.

6.2.7. d-ROM test

In this study, D-ROM test was used to estimate the total amount of oxidative metabolites of each sample. At the end of the study all samples were processed in a blind fashion.

6.2.8. Mitochondrial membrane potential and superoxide production

Peripheral blood mononuclear cells (PBMC) were isolated following standard procedures by density gradient centrifugation with Ficoll (Ficoll-Paque PLUS, GE, UK) from an aliquot of heparinised blood collected at each study visit. Mitochondrial oxidative stress production and membrane potential were assessed by flow cytometry using the mito probe MitoSOX Red (Invitrogen, UK) and 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen), respectively as described in previously described in the Methods section of this thesis.

6.2.9. HEME H0-1 ELISA

Plasma HO-1 concentrations were determined from 100 µL of plasma using an HO-1 (human) enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Farmingdale, NY, USA). This assay can detect HO-1 in the range of 0.78–25 ng/mL of undiluted plasma.

6.2.10. Statistical analysis

All data is presented as mean and standard error of the mean unless specified. Demographic data at baseline was compared between groups (RIPC versus placebo) at baseline by paired *t*-Test for numerical variables and Fisher's exact for categorical variables. Changes in FMD 24 hours after IPT were the primary outcome. Changes in all biomarkers were analysed as secondary outcomes using ANOVA for repeated measures (with post-hoc Bonferroni corrections when between groups comparisons were performed). For those variables with a statistical significant difference between

groups at day 1, a relative increase was calculated as follows: day 1 serum concentrations minus baseline, divided by baseline and multiplied by 100. Comparison of relative increases at day 1 between groups was performed by t-test or equivalent non-parametric method. Data were graphically tested for normality and logarithmic or square root transformations were made as needed before applying the adequate non-parametric tests. Univariate analyses were performed with crude values of each outcome variable between study groups at different time points. Further multivariate models were constructed including variables that showed a statistically significant association with the outcome from univariate analyses and they included age, gender, smoking, body weight and ethnicity. All analysis was performed with the statistical software package SPSS 22 (SPSS Inc. Chicago, IL, USA).

A minimum of 22 patients per group were needed to demonstrate a 2% difference in FMD between groups after 24 hrs (90% power, α 0.05, standard deviation of 1.6 (Tonetti et al. 2007)). A final sample of 24 participants per group was planned including a 10% drop-out rate.

6.3. RESULTS

From April 2013 to December 2013, 64 patients met the inclusion criteria for the study. 49 accepted to be enrolled into the study and underwent randomization of whom 47 completed the trial. An intention to treat analysis was performed including 24 patients in the Placebo Group and 23 patients in the RIPC Group (Figure 34). The patients' baseline characteristics were similar in both groups, recruited individuals were middle aged, 60% Caucasians, equally distributed between genders, slightly overweight and with 25-30% of current smokers (Table 28).

Table 28 Baseline charactheristics

	Placebo (N=24)	RIPC (N=23)
Age	47±9	45±9
BMI	26.5±3.8	26.1±3.7
Gender, Male	14(56.0%)	11(45.8%)
Ethnicity, Caucasian	15(60.0%)	15(62.5%)
Smoking,	7 (28.0%)	8 (33.3%)
Systolic BP, mmHg	120±16	118±10
Diastolic BP, mmHg	77.58±8.51	76.43±8.08
FMD, %	6.28±2.56	6.28±3.68
GTN, %	17.40±7.14	19.52±7.64

No serious adverse events were reported during the study. All study participants did not report any major changes in lifestyle for the entire duration of the trial. Baseline periodontal and multiplex parameters are described in Table 29 and 30.

Table 29 Periodontal baseline data

	Placebo (N=24)	RIPC (N=23)
PPD, mm	4.16±.82	3.84±.56
REC, mm	.85±.86	.86±.82
NPKTS, n *	69.33±28.96	61.00±21.81
FMPS, % §	63.97±16.23	58.90±15.6
FMBS, % ¶	49.86±21.97	50.05±16.04
NTEETH, n	28.46±2.72	28.57±2.84

FMPS: full mouth plaque score, FMBS: full mouth bleeding score, PPD: periodontal pocket depth, REC: gingival recession, NPKTS, number of pockets, NTEETH, number of teeth.
¶ Scores for full-mouth gingival bleeding were calculated for each patient as the number of sites with gingival bleeding on probing divided by the total number of sites per mouth, multiplied by 100.

*§ Scores for full-mouth plaque were calculated for each patient as the number of sites with detectable plaque divided by the total number of sites per mouth, multiplied by 100.**
** Periodontal pockets identified as sites with probing greater or equal to 5 mm*

Table 30 *Multiplex baseline data*

	Placebo (N=24)	RIPC (N=23)
IL-6, pg/ml	1.15 (0.63-1.79)	0.92(0.38-2.00)
IL-8 pg/ml	11.22±3.45	11.64±4.47
TNF-alpha pg/ml	4.00±1.92	3.52±1.85
E-Selectin pg/ml	21.26±16.43	20.75±15.20
P-Selectin pg/ml	122.89±62.12	114.92±43.91
s-ICAM3 pg/ml	1.92±3.86	2.02±3.39
Thrombomodulin pg/ml	5.30±3.98	5.47±6.06

6.3.1. Vascular function

FMD was lower in the Placebo group at 24 hours after IPT when compared to RIPC (unadjusted difference of 2.11%; 95% CI, 0.44-3.78; p=0.015). This difference disappeared at 7 days after IPT (unadjusted difference of 0.89; 95% CI, 0.97-2.74; p=0.339) (Figure 36). A 30% reduction of FMD was noted in the placebo group 24 hours after IPT when compared to RIPC (Figure 37). A greater reduction in endothelium independent vasodilatation (GTN) was further noted 24 hours after IPT in the placebo group when compared to RIPC (unadjusted difference of 4.77; 95% CI, 0.76-8.78; p=0.021), (Figure 38).

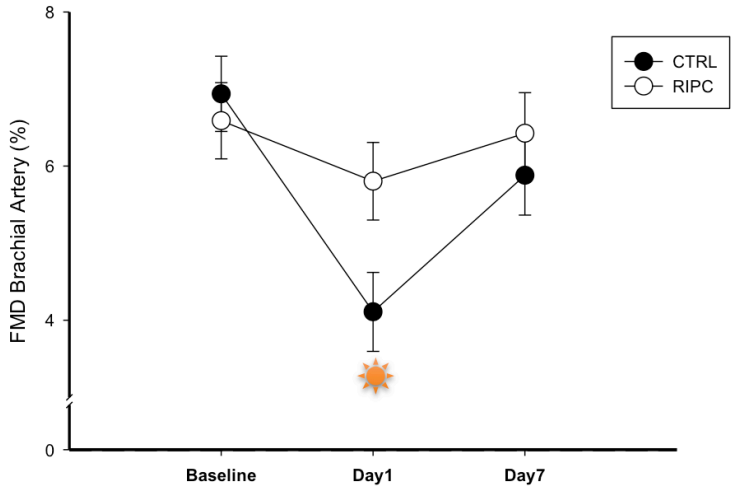


Figure 36 *Flow mediated dilatation during the study duration*

I bars represent SE. Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. FMD % changes, asterisks indicate significant between-group differences (P<0.001). **.** *Data adjusted for age, gender, smoking, body weight and ethnicity***.**

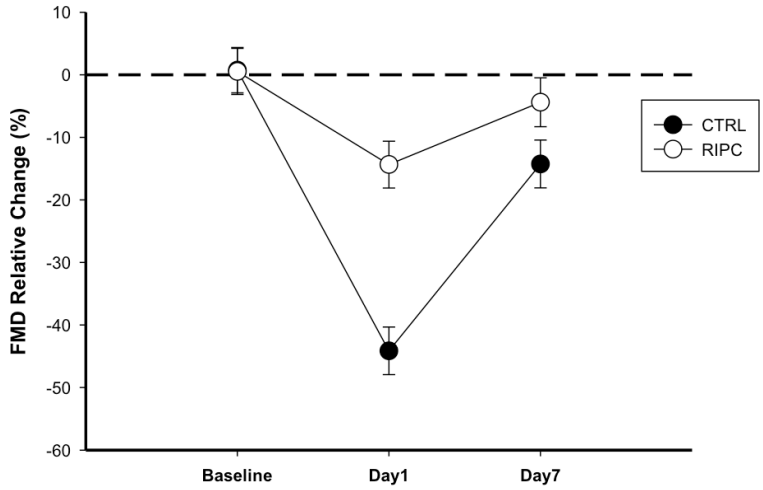


Figure 37 *Flow mediated dilatation relative changes during the study duration*

I bars represent SE. Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. FMD relative % changes.

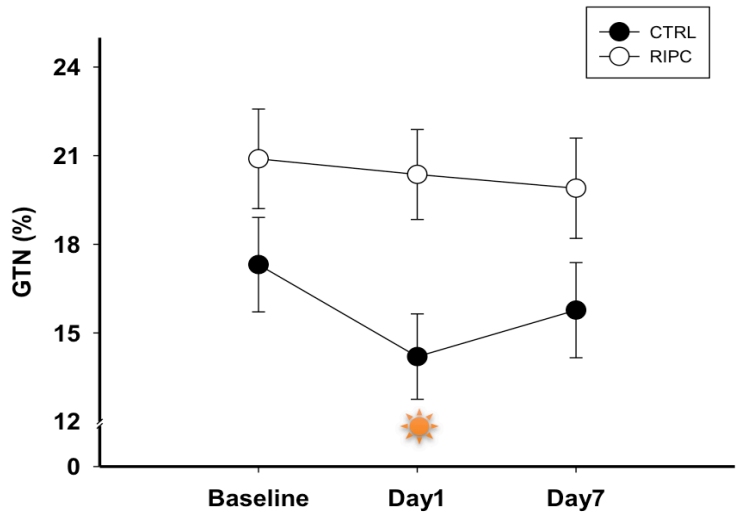


Figure 38 *GTN mediated dilatation relative changes during the study duration*

I bars represent SE. Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. GTN % changes, asterisks indicate significant between-group differences (P<0.001). **.** *Data adjusted for age, gender, smoking, body weight and ethnicity***.**

6.3.2. Markers of inflammation and vascular activation

Among all inflammatory biomarkers, repeated ANOVA analysis confirmed a statistically significant difference in CRP and TNF-α between groups over time. Similarly all endothelial markers were statistically significant different between groups. In particular patients in the RIPC group exhibited lower CRP level at day 1, and lower TNF-α at day 1 and day 7 after IPT when compared to placebo group patients ($p<0.05$ for all comparisons), (Figure 39). An inverse trend of acute release of endothelial markers was noted following IPT between groups. Indeed patients in the RIPC group presented with lower s-Eselectin at 24 hrs, P-selectin at 7 days, lower s-ICAM3 both at 24 hours and 7 days after IPT when compared to placebo group patients. Thrombomodulin levels between study groups although different at baseline, changed following an opposite trend. Patients in the RIPC group showed statistically significant lower levels of thrombomodulin at 7 days after IPT when compared to placebo patients (Figure 40). Non adjusted data presented in Table 31 and 32.

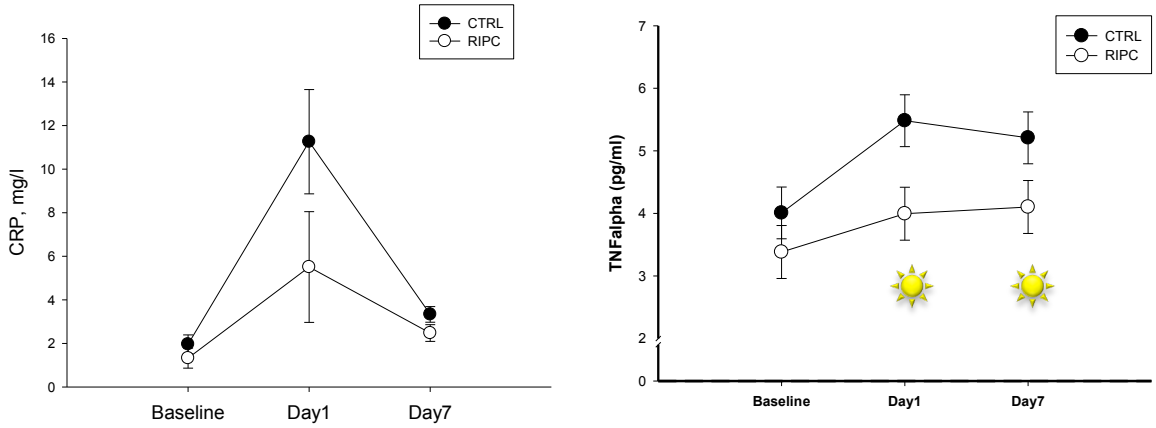


Figure 39 *Inflammatory mediators during the study duration*

I bars represent SE. Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. Inflammatory markers changes, asterisks indicate significant between-group differences (P<0.05).

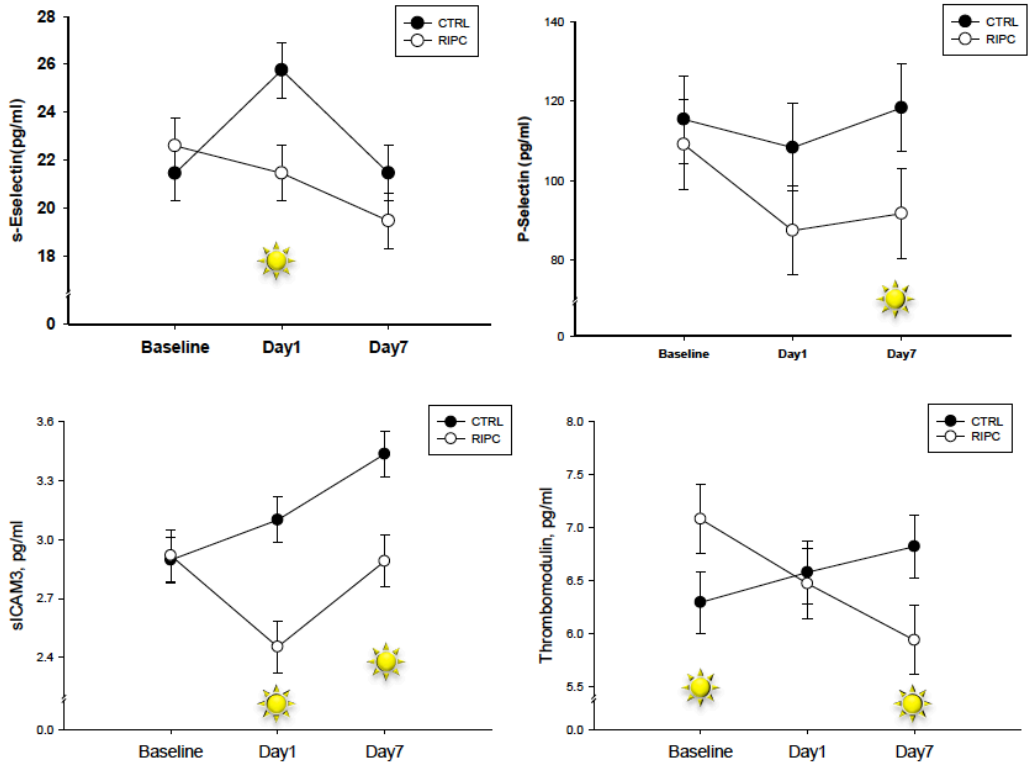


Figure 40 *Vascular markers during the study duration*

I bars represent SE. Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. Asterisks indicate significant between-group differences (P<0.05). Data adjusted for age, gender, smoking, body weight and ethnicity.

Table 31 Non adjusted between group differences of markers of inflammation an vascular activation at Day 1

Marker	Unadjusted between group difference at Day 1	95% CI	p value
CRP	5.55	0.36-11.47	0.035
IL-1	-1.19	-2.63-0.25	0.096
IL-6	0.58	-1.04-2.21	0.474
IL-8	1.63	-2.29-5.54	0.408
IL-10	4.35	-0.05-8.76	0.053
IL-12	1.29	0.15-2.42	0.028
TNF-α	1.34	0.11-2.79	0.041
IFN-γ	9.95	0.44-19.46	0.041
E selectin	5.96	-4.51-16.43	0.257
P selectin	22.55	-8.47-53.57	0.15
Thrombomodulin	0.28	-2.80-3.37	0.854
ICAM 3	0.29	-1.84-2.42	0.784

Table 32 *Non adjusted between group differences of markers of inflammation an vascular activation at Day 7*

Marker	Unadjusted between group difference at Day 7	95% CI	p value
CRP	0.87	0.13-1.86	0.086
IL-1	-0.15	-0.63-0.34	0.511
IL-6	0.52	-0.74-1.78	0.411
IL-8	0.94	-2.01-3.89	0.525
IL-10	0.29	-5.44-6.02	0.919
IL-12	-4.81	-11.73-2.12	0.161
TNF-α	0.96	0.25-2.17	0.03
IFN-γ	-1.17	-8.31-5.96	0.737
E selectin	3.65	-6.12-13.43	0.455
P selectin	28.31	0.32-61.93	0.04
Thrombomodulin	0.84	0.16-3.85	0.044
ICAM 3	0.19	-2.33-2.71	0.879

6.3.3. d-ROM test

Patients in the placebo group exhibited higher levels of reactive oxygen metabolites at baseline and no other differences were noted between groups over time (P<0.001). When analysing the relative changes in d-ROM % after day 1 and day 7 following

therapy, patients in the RIPC group presented with approximately 30% more reactive oxygen metabolites compared to the patients in the placebo group (Figure 41).

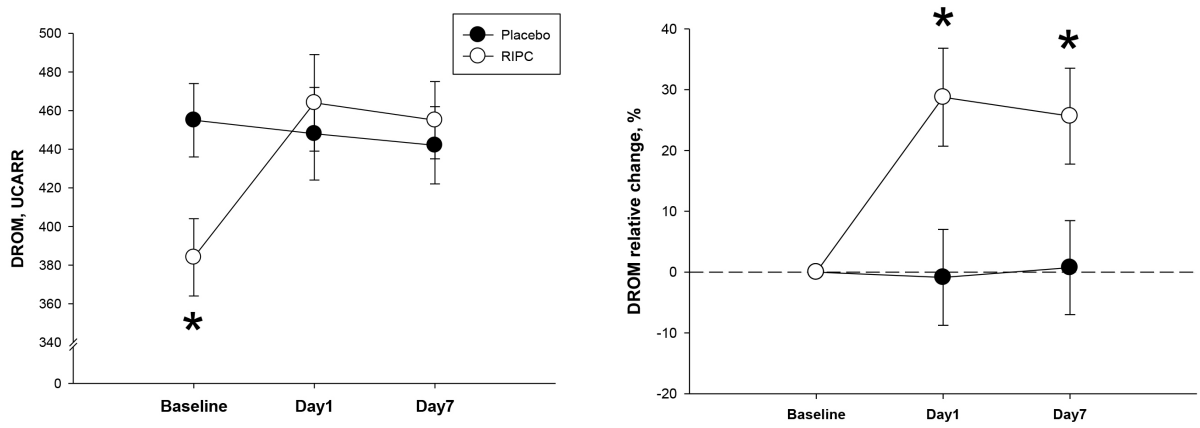


Figure 41 *Reactive oxygen metabolites during the study duration*

I bars represent SE. Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. Asterisks indicate significant between-group differences (P<0.05).

6.3.4. Mitochondrial ROS production and membrane potential

PBMC isolated in the RIPC group had a non-statistically significant higher mtROS production compared to those in the placebo group 24 hours following dental treatment (unadjusted difference of -5.30; 95% CI, -17.35-6.75; p=0.380) (Figure 42). When subpopulations of PBMC were analysed separately, the increased production of mtROS from PBMC was mainly due to a higher superoxide production in lymphocytes (unadjusted difference of -4.23; 95% CI, -10.25-2.30; p=0.198). Further a non-statistically significant trend of increased oxidative stress was also detected in monocytes (unadjusted difference of -0.93; 95% CI, -45.35-43.48; p=0.966) (Figure 41). No differences between groups in the mitochondrial membrane potential of PBMC (unadjusted difference of 1.07; 95% CI, -1.22-3.77; p=0.349), lymphocytes (unadjusted difference of -0.33; 95% CI, -1.81-2.46; p=0.759) and macrophages (unadjusted difference of -0.08; 95% CI, -0.50-0.34; p=0.694) 24 following RIPC were observed.

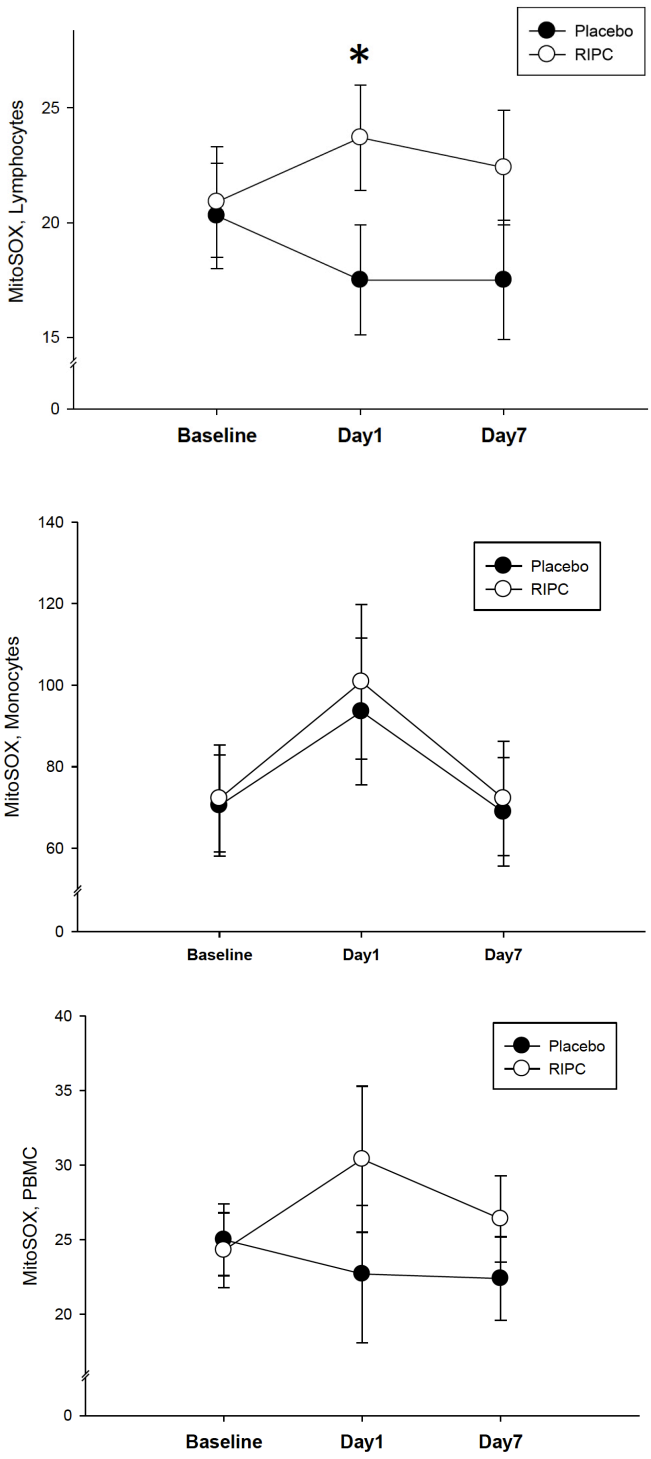


Figure 42 *Changes in mtROS production during the study period*

6.3.5. LPS

Patients in the RIPC and placebo group presented with similar plasma LPS levels at their baseline visit. 24 hours following therapy, no substantial differences were detected in LPS profiles between the two study groups (unadjusted difference of 0.07;

95% CI, -0.44-0.58; p=0.775). Both groups exhibited a reduction in LPS plasma levels following one week of periodontal treatment.

6.3.6. Heme oxygenase 1

No statistically significant differences were detected in HO-1 levels at baseline and overtime between the two study groups (day 1 unadjusted difference of 14.67; 95% CI, -8.63-37.96; p=0.211), (day 7 unadjusted difference of 8.13; 95% CI, 9.04-25.29; p=0.344) .

6.4. Discussion

This study demonstrated that in patients suffering from periodontitis, 24 hours following intensive periodontal treatment, endothelial function was only mildly impaired when preceded by RIPC when compared to placebo. This benefit on the endothelium was associated with a reduction of all soluble markers of endothelial cell activation and some common inflammatory markers. Some of these differences were still present 1 week after IPT, although vascular function had returned to normal. An increased oxidative stress response was also observed parallel to the decreased vascular function. These findings corroborate the impact of systemic inflammation on vascular function and provide evidence on the possible mechanisms behind the vascular benefit of RIPC.

Full mouth scaling and root planing (IPT) induced a transient inflammatory response and endothelial impairment as previously reported[12]. Acute impairment of vascular function was associated with increase in systemic inflammation and endothelial activation. Previous evidence suggested that RIPC is protective for the vasculature via two different windows[577]. The earliest phase of protection is active within 4 hours following RIPC, the second and longer protective effect has been identified between

24 and 72 hours. As the IPT was initiated within 30 minutes from preconditioning, it is plausible to consider that the beneficial effects on the vascular endothelium observed in this study, related to the first window of protection.

RIPC has been previously used in human models in order to prevent endothelial dysfunction following different inflammatory stimuli (typhoid vaccination, strenuous exercise) and it has shown a protective effect towards the endothelium[716]. This is the first trial that explores its effects on markers of inflammation and endothelial activation following IPT. The changes in some serum inflammatory markers observed in this study suggest that the protective effect of RIPC on the endothelium might be partially mediated by the modulation of systemic inflammation. Similarly we could infer that the inflammatory mediators profile could be responsible for the endothelial impairment observed 24 hours following the dental treatment. Substantial changes in endothelial activation markers between the RIPC and placebo groups were noted. Macromolecular adhesive associations between cells are important for transmitting spatial and temporal information that is critical for immune system function. Among such group of proteins, the intercellular adhesion molecules (ICAMs) have multiple functions including intracellular signaling events[717]. The normal expression pattern of ICAM3 is high on all leukocytes but lacking on endothelium. It is likely that this molecule plays an important role in early events during leukocyte-leukocyte contact including B cell activation mediated by T cell help or T cell activation by antigen-presenting cells (APCs). Monoclonal antibodies (MAbs) to ICAM-3 have been shown to inhibit peripheral blood lymphocyte proliferation in response to phytohemagglutinin, allogeneic stimulator-cells, and specific antigens[718].

Endothelial activation often precedes endothelial dysfunction. Differential cell surface molecule expression between quiescent and activated endothelial cells influences not

only the relative balance between pro- and anti-coagulant activity, but also the degree of adhesion of circulating blood cells. E-selectin is expressed on activated endothelial cells, where, in combination with P-selectin, it facilitates rolling of leukocytes along the endothelial layer as a prelude to leukocyte adhesion (facilitated by the upregulation of ICAM-1 and VCAM-1 [vascular cell adhesion molecule-1]) to activated endothelial cells and subsequent transmigration across the endothelial barrier to a site of injury or inflammation[719]. Given their specificity for endothelial cells in the activated state, soluble forms of these cell-surface molecules, shed from endothelial cells after activation, have been widely studied as diagnostic and prognostic markers in a variety of infectious diseases. Thrombomodulin (TM) is present in large quantities on the surface of the endothelium, particularly in the microcirculation, where it acts as an anticoagulant[720]. The TM-thrombin complex catalyses the formation of the anticoagulant molecule activated protein C, and prevents thrombin from converting fibrinogen to fibrin and from exerting other pro-coagulant effects. Consistent with the pro-coagulant properties of the activated endothelium, cell-surface thrombomodulin expression is reduced during sepsis. This phenomenon is likely to be linked to secondary to shedding of the molecule. Soluble TM (sTM) has therefore been proposed as both a diagnostic and prognostic marker of endothelial activation/dysfunction[721]. Lower serum levels of s-ICAM3, sE-Selectin and sTM, together with the reduction of TNF and CRP, reported 24 hours after RIPC and IPT suggest that RIPC could act as a modulator of the immune system and subsequently of endothelial activation. These properties could explain the differences observed in vascular function assessed by FMD in our study. For the first time, we have analysed Heme oxygenase 1 (HO1) concentration in plasma in a human model of acute systemic inflammation. HO1 is an inducible heat shock protein that seems to be involved in the

protection from ischemia-reperfusion damage provided by RIPC. In our study we did not detect a difference in the (HO1) profile between test and control groups. An alternative mechanism could have been mediated on the immune response to the acute bacterial burden that the invasive dental treatment could have produced. In our trial we have not detected substantial differences in LPS levels between the study groups. This finding could rule out a potential action of RIPC on the systemic bacteremia following IPT. However, the time points in which we have obtained blood samples could not be the most adequate to analyse the relationship between RIPC and bacteremia. Further, assays measuring LPS circulating levels are rather not-specific (gut versus oral microflora). We could speculate that a lower level of inflammation could have reduced the permeability of blood vessel in the periodontium reducing the amount of bacterial dissemination. There is increasingly interest on the impact of invasive dental procedure both with regards to the long-term risk of vascular diseases as well as on the myocardial tissues (risk of endocarditis). Evidence from an observational study suggests an increased risk of CV events in the 4 weeks following invasive dental procedures[519]. Therefore, reducing the amount of acute endothelial dysfunction could be beneficial in populations at high risk for CVD. Our data have also associated RIPC with a higher level of mitochondrial superoxide production and a potential higher level of systemic oxidants. A potential explanation for this finding could be related to the production of ROS during the ischemia-reperfusion cycles provoked by the RIPC. It is now accepted that ROS could be generated in both phases of RIPC and not only following reperfusion as initially proposed[722]. The concept of a detrimental role of ROS should also be carefully evaluated. Vanden Hoek et al. reported the loss of preconditioning protection treating in cardiomyocytes with antioxidants[723,724]. We have reported the increase in ROS production both in PBMC

mitochondria and whole blood in a human model. Therefore this increase could be potentially partially involved in the explication of the protective effect of RIPC. However, a more detailed assessment of the oxidative status following RIPC should be investigated including more in depth and specific measure of oxidative stress over time. In addition, we recognize a limitation in our study. Indeed in support to our finding, an additional control group could have been recruited. Individuals not undergoing periodontal treatment could have been recruited and assessed for changes in Mitosox and d-Rom levels after 24 hours and one week.

Although this study is the first one to investigate possible mechanisms underlying the transient effect of IPT on the vasculature there are some limitations to consider. This study included a small group of individuals and the results might not be valid for all patients suffering from periodontitis as well as following other dental invasive procedures (i.e. tooth extraction or surgical procedures). Although we included healthy individuals with no other systemic conditions known to impact on the endothelium such as hypertension, heart failure, atherosclerosis, hypercholesterolemia, diabetes mellitus, smoking and aging, we cannot rule out an alternative mechanism of protection of RIPC on vascular dysfunction. Strengths of the study though include the adoption of a validated flow mediated dilatation protocol and using a single trained vascular examiner masked to the group allocation, who performed and analysed all the scans with a standardized protocol. In addition, a single clinician performed all the IPT blind to the patients' randomization.

6.5. Conclusion

In conclusion intensive periodontal therapy is associated with acute impairment of vascular function associated with systemic inflammation and endothelial cell activation. RIPC performed 30 minutes before IPT can prevent both acute inflammation and endothelial dysfunction. Further research is needed to ascertain whether RIPC could be performed when IPT or other invasive dental procedures are performed in individuals with known comorbidities and increased vascular risk and whether RIPC could produce a similar benefit.

7. FINAL DISCUSSION

7.1. Summary of the main findings

This thesis aimed to address the impact of the chronic exposure to inflammation and oxidative stress on the human vasculature homeostasis observed in patients with periodontitis and investigate biologically plausible mechanisms underlying this link.

Specifically, this project analyzed the relationship between PD and two recognized measures of vascular health and potential indicators of a future cardiovascular risk: the endothelial function, involved in the very early stage of the atherosclerotic disease pathogenesis and the c-IMT, a measure of structural arterial disease linked to atherosclerotic progression. The results of the systematic review and meta-analysis supported this association; PD could contribute to a faster progression of the c-IMT leading to the development of atherosclerosis and increased risk of future cardiovascular events. This was further confirmed by an additional and independent systematic review and meta-analysis by Zeng et al. on 17330 participants. They showed that periodontitis is associated with carotid atherosclerosis (OR: 1.27, 95% CI: 1.14-1.41; P<0.001)[725].

The current epidemiological findings of a moderate association between PD and atherosclerosis do not allow defining PD as an independent casual factor in the onset and progression of atherosclerosis. Reviewing the modified Hill's criteria to explore causation, we acknowledge that PD satisfies the majority of the criteria (Table 33).

Table 33 Bradford-Hill criteria for the causal association between PD and CVD

Bradford-Hill Criteria	
Statistical strength of association	The strength of association between PD and CVD is considered weak to moderate.
Consistency	The association between PD and CVD is consistent among a large number of studies. However, consistency of the findings of available studies is not absolute.
Specificity	Positive association between periodontal and cardiovascular diseases was evidenced adjusting for traditional cardiovascular risk factors
Temporal relationship	PD preceded CVD after adjustment for traditional cardiovascular risk factors.
Biological gradient	Increasing severity of PD resulted in higher cumulative incidence of CVD after adjusting for potential confounders.
Biological plausibility	Experimental evidence proves the biological plausibility of a causal association between PD and CVD.
Coherence	The association does not conflict with currently established theory and scientific knowledge on the development of CVD.
Experimental reversibility	*In vitro* and *in vivo* evidence supports a causal role for PD in the development of CVD. However, RCT on hard CV outcomes, ie MI and stroke, need to be designed.
Analogy	Other inflammatory conditions such as diabetes can induce CVD in humans. PD can induce CVD in animal models.

Despite the overall modest association, the consistency of data across different study populations, exposures and outcome variables suggests that these findings might not be spurious or attributable to confounders. There is lack of experimental evidence on the reversibility of the association obtained from systematic review of randomized controlled clinical trials testing the hypothesis that control of PD will result in a stop or reversion of atherosclerosis.

Multiple plausible mechanisms have been suggested to support the causality of the association. Periodontal lesions, characterized by an exaggerated inflammatory gingival response and interface with a dysbiotic microflora, represent a constant source of transient systemic bacteraemia[665]. Periodontal pathogens or their byproducts gaining access to the circulation could trigger a systemic inflammatory response involving the endothelium and immune cells (Figure 43).

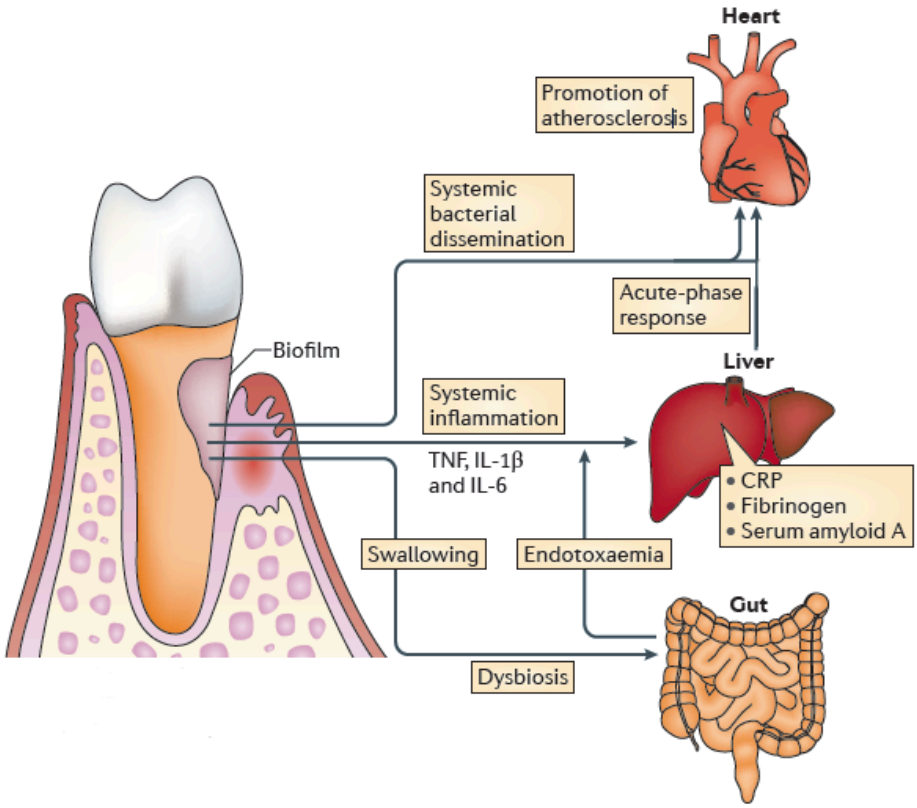


Figure 43 *Mechanisms of causal association adapted from Hajishengallis et al. 2015*

This mechanism has been demonstrated plausible in experimental animal models of periodontitis and atherogenesis[726-728].

Further, evidence suggests the necessity of pattern-recognizing receptors for atherosclerotic lesion formation[729-733]. TLR2 recognition of Porphyromonas gingivalis is fundamental for the impact of the pathogen in the periodontium[734,735] and in atherosclerosis[325,736]. P. Gingivalis has been shown to invade the endothelial cells and potentially induce endothelial dysfunction[737,738], a key step in the development of atherosclerosis[739]. Circulating endothelial progenitor cells (EPC) contribute to the maintenance of a healthy endothelium[740] and their number has been related to both endothelial function and CV outcomes[741,742]. Li et al has reported an increased EPC counts in otherwise healthy periodontal patients compared to controls without periodontitis[516] and that periodontal treatment was related to a decrease of CD34 positive cells[743]. In an experimental animal model of periodontitis, it has been reported that recurrent bacteraemia induce endothelial progenitor mobilization from the bone marrow and subsequent higher levels of EPC[744], a potential counter-measure against the bacteraemia-mediated endothelial damage. Similar findings are also detectable in other inflammatory conditions such as rheumatoid arthritis[745], or in acute tissue damage events, such as MI[746] or percutaneous coronary intervention[747].

An alternative causal mechanism has been suggested by a recent experiment on mice reporting that P. gingivalis can cause alterations to the gut microbiota, leading to indirect induction of systemic inflammation[748]. Mice orally infected with P. gingivalis showed changes in their gut microbiota with increased proportion of Bacteroidetes and a decreased proportion of Firmicutes compared to controls. The gut micriobiota shift correlated with the decreased expression of tight-junction proteins in the ileum and with the onset of endotoxaemia and systemic inflammation. However, the

mechanism by which P. Gingivalis causes changes to the gut microbiota remains still uncertain.

Our meta-analysis has confirmed both an impaired endothelial function in patients with PD and a beneficial effect of periodontal treatment on the same marker in clinical trials involving otherwise healthy patients. Our group previously conducted a RCT on otherwise healthy patients with severe and generalized PD reporting an improvement of endothelial function, assessed by FMD, 6 months after periodontal treatment[12]. In the review, we could not draw robust conclusions on the effect of periodontal interventions on c-IMT since our search did not find reliable evidence published.

Piconi et al. conducted an uncontrolled 12months cohort clinical trial and concluding that periodontal therapy has a favourable effect on c-IMT progression. Due to its low level of evidence, however no firm conclusions can be drawn [399]. Recently, Kappelas et al. conducted a RCT on 168 Aboriginal Australians suffering from PD and observing a c-IMT decrease after 12 months of a single session of periodontal therapy in the intervention group (mean reduction=−0.023 [95% CI, −0.038 to −0.008] mm) but not in the control group (mean increase=0.002 [95% CI, −0.017 to 0.022] mm). The difference in intima-media thickness change between treatment groups was statistically significant (−0.026 [95% CI, −0.048 to −0.003] mm; P =0.03)[652]. However, the participants received a single session of periodontal therapy with a lack of SPT sessions and reported modest periodontal improvements. In addition, all participants were free to receive periodontal treatment during the course of the study. Furthermore two loops were obtained from each side of the carotid, and averaged to obtain the maximum carotid IMT increasing the variability of the measurements. The Oral Infections and Vascular Disease Epidemiology Study (INVEST) has documented that higher levels of periodontopathogens were cross-sectionally associated with thicker

carotid IMT[594]. In addition, the same investigators reported longitudinal change in periodontal health to be concurrent with longitudinal carotid artery IMT progression over an average period of 3 years[749]. Desvarieux et al. detected a difference in c-IMT among participants of approximately 0.1 mm in 3 years follow-up suggesting the importance of the improvement in periodontal status. Evidence suggests that a 0.03 mm/year increase in c-IMT is associated with a 2.3-fold increased risk for CV events[648]. In addition, experimental studies on the impact of statins on the c-IMT progression rate reported as clinically significant a difference of 0.0082 mm/year in c-IMT between test and controls[538]. Our meta-analysis, in line with previous evidence, supports the role of PD as a contributor to the pathogenesis of CVD and indicates the endothelium as the main target of the systemic effect of PD.

We designed 3 RCTs to explore the nature of the association between PD and atherosclerosis including the first in vivo exploration of plausible underpinning mechanisms. In Study 2, we randomized patients with PD and T2DM to receive either sub-gingival scaling and root planing and where appropriate Modified Widman flap (as described in Chapter 2) or supra-gingival scaling and polishing over a period of 6 months and adopted FMD to analyzed changes in endothelial function in the two study groups. The choice of a high CVD risk population was justified by the importance of both diseases (PD and CVD) in patients with diabetes. A wealth of evidences link both T1 and T2DM to a deterioration of the endothelial function showing an impaired endothelial-dependent vasodilation[750-752]. Despite many proposed mechanisms for this relationship, the definitive pathogenesis remains unclear; multiple homeostatic imbalances and hyperglycemia suggest a multifactorial etiology[753-755]. The chronic inflammatory state observed in T2DM including higher levels of pro-inflammatory cytokines, chemokines, and adhesion molecules could affect the endothelial cells

phenotype[756]. The adipose tissue has been identified as the major source of inflammatory mediators such as TNF-α[757,758] that activates nuclear factor κB (NFκB), a transcription factor inducing the expression of inflammatory genes[759]. In addition, overexpression of either TNF-α or NFκB kinase could contribute to the development of insulin resistance[760]. The association of insulin resistance with inflammation could reduce NO bioavailability[761]. The oxidative status is also implicated in the reduced endothelial integrity in T2DM. The accumulation of ROS such as superoxide anion (O_2^-) could diminish NO bioavailability, either through direct degradation by ROS, or alterations in the functional capacity of eNOS[762]. Xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondria represent important sources of O_2^-[762]. Mitochondria produce negligible ROS in physiological circumstances but high glucose concentrations increase the proton gradient within the electron transport chain with a consequent overproduction of superoxide[763]. Furthermore, mitochondrial dynamics such as increased mitochondrial fission and fragmentation could affect ROS production in DM[764]. Altered mitochondrial function is linked to several acute and chronic inflammatory diseases[641]. The mitochondrial respiratory chain is the main source of ROS [765]. Furthermore, mitochondria are also targets of oxidants. Under physiological conditions, about 1 to 3% of molecular oxygen is incompletely reduced during redox reactions in the mitochondrial respiratory chain leading to the production of the superoxide anion. However, under pathological conditions, an excess superoxide causes the activation of redox-sensitive transcription factors such as nuclear factor-κB and a subsequent increase in the expression of cytokines, chemokines, eicosanoids, inducible nitric oxide synthase (iNOS), and adhesion molecules[766]. Several inflammatory molecules such as TNF-α and IL-1β and the reactive nitrogen intermediate NO, may induce mitochondrial damage[767-769]

decreasing the activity of the respiratory chain, ATP production and mitochondrial membrane potential. In addition, these mediators induce the accumulation of significant amounts of ROS[770,771]. This project for the first time proposes PD as an underestimated factor driving systemic inflammation and oxidative stress in patients with T2DM and driving their increased risk of macrovascular complications. The improvement of the endothelial function was associated with the lowering of the mitochondrial superoxide production, IFN-γ, TNF-α, and s-Eselectin. Superoxide overproduction is a mediator of tissue damage in T2DM; it activates multiple pathways involved in the pathogenesis of complications and inactivates protective enzymes such as eNOS and prostacyclin synthase[772]. The pro-inflammatory cytokine IFN-γ is secreted by T helper-1 (Th1) cells during infections. IFN-γ can subsequently activate macrophages, resulting in the secretion of inflammatory mediators to enhance antibacterial actions. Endogenous pro-inflammatory mediators, such as tumor necrosis factor (TNF), may also induce the production of IFN-γ. IFN-γ stimulates the endothelium to express cell adhesion molecules and leukocyte recruitment to the plaque[773]. In addition, the presence of LPS can increase the process[774]. Therefore, the beneficial effect of the periodontal treatment reported in our sample might be justified by a minor level of bacterial dissemination associated with improvement of the periodontal health. This is consistent with the findings of a reduced LPS levels observed within 6 months of periodontal therapy. However, our analysis does not allow discriminating the source of LPS detected. More in depth analysis on the potential oral source of the bacterial toxins should be pursued in the future.

In study 3, we have evaluated the impact of periodontal treatment on c-IMT in a RCT on 117 patients suffering from periodontitis and T2DM reporting a different trend in the progression of the carotid thickening between the two groups. Our results suggest

that the chronic inflammatory stimulus of PD induces structural changes of the vascular wall reflected by progressive increased thickness of the intima media layer[775,776]. Therefore changes in cIMT might be a marker of the chronic vascular inflammation and oxidative stress burden in each individual.

Study 3 reported an improvement in endothelial function in T2DM, which in turn could represent a clinically relevant benefit in these patients and possibly lowering their CVD risk. Indeed, we observed an absolute difference of 0.9% (95% CI, 0.3-1.4; p=0.002) in FMD after 12 months of periodontal therapy. Several prospective studies reported an inverse association between brachial FMD and CVD risk[777-780] although some evidence do not support this association[781,782], particularly in the asymptomatic population[783-785]. Hence the diagnosis of co-morbidities may impact the relationship between FMD and CVD. A meta-analysis of 14 prospective studies reported a 13% lowering of future CVD per every 1% increase in FMD[786]. This finding corroborates the previous conclusion of our group[12] and report an improvement of the vascular health not only in cases of severe, generalized periodontitis but also in moderate forms of periodontal infection.

Further evidence of an intimate relationship between PD and vascular function (assessed by FMD of the brachial artery) comes from the observed acute vascular dysfunction following periodontal intensive treatment. Our group reported a consistent transient endothelial dysfunction 24 hours following IPT associated with an increase of systemic markers of inflammation [12]. This further corroborates the role of inflammation in driving atherogenesis and its complications. PD is a common source of local and systemic inflammation. We recently confirmed that the degree of inflammatory exposure periodontal therapy represents is linked to the extent of periodontal therapy performed[787].

Further, this evidence poses an additional question, which is whether acute inflammation following invasive dental treatments could be detrimental on patients' overall homeostasis and whether this could be prevented. Our group was the first to report on the possible association between dental treatment and acute increase in vascular risk. Minassian et al. adopted the self-controlled case series method to investigate the incidence of acute CV events such as ischemic stroke and myocardial infarction following invasive dental treatment by using Medicaid claims data from the United States. The authors reported "invasive dental procedures may be associated with a transient increase in the risk for stroke and myocardial infarction in the first 4 weeks after treatment"[519].

Lower FMD values have been associated with a higher risk of CV events. In Study 4 we therefore we chose a simple and effective procedure of vascular protection: RIPC. This has been elegantly demonstrated in different experimental models of systemic inflammation such as the Typhoid vaccination, ischemia-reperfusion injury and strenuous exercise[297,577,716]. For the first time we experimented whether RIPC just performed before a long session of intensive periodontal therapy would prevent or reduce the acute endothelial dysfunction observed following IPT. The results demonstrated in the patients recruited, that RIPC modulates the acute inflammatory response following IPT and resulting in increased FMD compared to the placebo group. Although RIPC could not prevent endothelial dysfunction, a significant 30% difference was observed between the two study groups. This could represent a relevant finding in patients with known co-morbidities or unstable medical conditions who require essential, emergency or elective dental care. Further research is warranted with particular emphasis on the possible implications on the provision of dental care in special care units.

Study 4 concluded that changes in mtROS production in PBMC and whole oxidant status of patients undergoing IPT might relevant to the development of vascular dysfunction. In particular the increase in oxidative circulating molecules might exert a vasculo-protective effect. Vanden Hoek et al. reported the loss of preconditioning protection with antioxidants in cardiomyocytes[723,724]. Isolated cardiomyocytes demonstrated significant preconditioning protection with exposure to a 10-min ischemic preconditioning trigger just before 1 h of ischemia and reperfusion. Oxidant generation was observed to occur during the brief 10 min of preconditioning ischemia that could be attenuated with antioxidants and mitochondrial inhibitors.

This finding is in apparent conflict with the reduced systemic release of pro-inflammatory mediators and vascular activation/damage molecules observed in patients receiving RIPC. Further, no substantial difference in LPS levels were noted and within the limitations of this assay, we can at least conclude that bacterial burden might be less important in the link between acute inflammation and vascular function/dysfunction. The mechanism by which intensive periodontal treatment triggers acute endothelial dysfunction seems to be attributable to a systemic inflammatory response. Increase in C-reactive protein and TNF are both involved in the amplification of the immune response. In our trial is also evident that there is a modulation of the vascular activation. The adhesion molecules levels increase and higher thrombomodulin (TM) suggest a direct effect on the endothelium. Whilst higher levels of vascular activation could account for an increased endothelial-leukocytes interaction, TM might be an expression of an acute endothelial damage following periodontal treatment. TM plays a pivotal role in endothelial homeostasis by inhibiting coagulation and fibrinolysis[788] and mediating inflammation[789]. This gives the endothelium protection against apoptosis, inflammation, fibrinolysis and

thrombosis[790]. The sample population recruited for Study 4 exhibited baseline low circulating levels of TM. Elevated plasma TM levels have been found to correlate with several clinical conditions such as atherosclerosis[791,792] and ischemic stroke[793]. Our data report an increase in sTM levels up to 7 days after invasive periodontal treatment. Using sTM as a marker of vascular damage, our project results could support the hypothesis that the systemic perturbation subsequent periodontal intervention might temporarily increase CV risk through endothelial dysfunction. Further research is required to confirm our result in a high risk population and also investigate if other invasive dental procedures such as single or multiple extractions and dental implants placement could represent a potential trigger for acute endothelial dysfunction.

7.2. Limitations

Our systematic search and meta-analysis, for the first time, summarized all the available evidence from observational and intervention studies on the relationship between PD and specific CV biomarkers. However, the relatively limited number of patients included, the lack of systematic adjustment for confounders and difference in the methodology adopted for the assessment of both the dependent and independent variables could represent significant limitation of Study 1. We detected a high level of heterogeneity in studies retrieved when gathering the data on FMD and PD due to the high variability in the FMD technique. In Study 2, our main limitation was the relatively low sample size and short follow-up (6 months) which might not be representative of the evolution of atherosclerosis. In addition, we adopted one technique for the assessment of PBMC mitochondrial ROS production. This is not necessarily the most sensitive technique for oxidative analyses. Similarly, in Study 4 we have used a generic measure of oxidative stress (d-ROM test) and FACS analysis for the mitochondrial

superoxide production. These methodological steps could have introduced bias in the interpretation of our results. In Study 3 although the sample size calculation based on recent evidence suggested sufficient power for detection of c-IMT changes, the majority of RCTs and evidences assessing the progression of atherosclerosis are for a minimum of 2 years of follow-up. This is also coupled with the lack of any evidence of changes in the occurrence in clinical vascular events which would represent the ultimate goal of this area of research.

7.3. Relevance of the project

This project contributed to the advancement in the understanding of the link between PD and CVDs. Adopting a systematic approach to the available evidence, we confirmed a moderate association between PD and CVD. Dentists and physicians should be more aware of this link. Furthermore in 2 RCT we have demonstrated that periodontal treatment has a beneficial effect on the endothelial regulation of the vessel tone in a high CVD risk population represented by patients with T2DM. In addition, the amelioration of the vascular health is expressed by a slower progression of the c-IMT thickening assessed within 12 months after the periodontal treatment in a population affected by T2DM. PD could represent a modifiable risk factor for endothelial dysfunction and the atheroma progression. Therefore, considering the prevalence of PD, its treatment could be relevant to the management of CVD in the general population. The most plausible mechanism linking the two conditions is represented by inflammation. Acute and chronic changes in inflammation and oxidative stress could influence this link.

Furthermore, we have proven the protective effect of RIPC towards the transient endothelial dysfunction observed within 24 hours from intensive periodontal therapy providing a potential tool in the prevention of CV risk in population with co-morbidities. Our research has increased the number of evidence suggesting a causal role of PD in the pathogenesis of atherosclerosis. Evaluating the effect of periodontal treatment on CVD hard outcomes such as stoke and MI should be the future goal for researchers on this topic.

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